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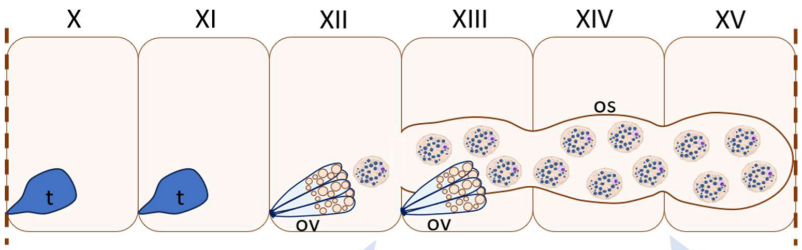
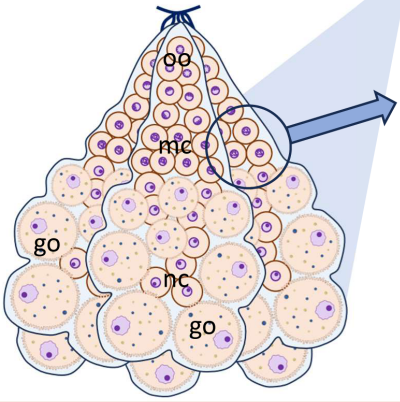
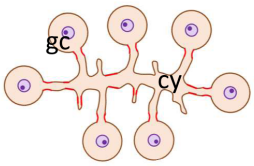


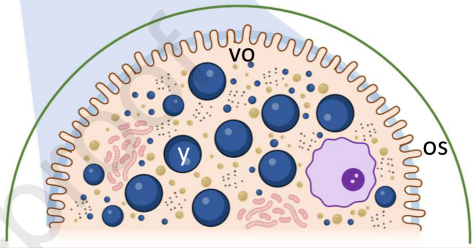

*Delaya* sp. possesses two pairs of testes (t) in body segments X-XI, two pairs of ovaries (ov) in segment XII-XIII, and two long ovisacs (os) extending from segment XII to XV.




Germ cells (gc), but not growing oocytes, are interconnected and form cysts equipped with reticular cytophore (cy).

The ovary is made up of several units, each with a narrow apical region containing oogonia (oo) and germ cells in meiotic prophase I (mc) clustered as germline cysts, and a broader distal region housing growing oocytes (go) and nurse cells (nc).




The ovisac (os) is well developed in specimens with active oogenesis and tightly packed with absorbing yolk oocytes (vo). *Delaya* sp. produces yolk-rich oocytes with abundant nutritive material (y).

# Ovary organization and oogenesis in two species of cave-living clitellate annelids from the genus *Delaya* (Clitellata, Pelodrilidae)

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**Abstract**

Clitellate annelids (Clitellata) are hermaphrodites with gonads localized in specific segments in the anterior body part. Localization of gonads and the structure of the reproductive systems are considered conservative traits of clitellate evolution and are used as crucial features in their taxonomy and in phylogenetic considerations. The study aimed to present the ovary morphology, histology, and ultrastructure in two *Delaya* species. The genus *Delaya* groups poorly known cave-living clitellate annelids, and their ovary organization and oogenesis are entirely unknown. Moreover, their taxonomic status is under debate. According to recent molecular analyses, *Delaya* and two other genera form the family Pelodrilidae, closely related to earthworms. To enhance our understanding of these cave-living animals' reproductive biology and provide new characters that may aid in phylogenetic considerations, the light and electron microscopic techniques were used to study the organization of the ovaries and the course of oogenesis in two species: one from a cave in Greece (*Delaya* sp. GR) and the other from a cave in France (*Delaya* sp. FR). In both species studied, two pairs of ovaries are located in two consecutive segments – XII and XIII. Each ovary consists of 3-5 functional units. The ovarian units are polarized: their apical parts (attached to the septum) contain oogonia and early meiotic cells, while the broader distal ends contain growing oocytes and nurse cells. Initially, germline cells (oogonia and early meiotic cells) develop synchronously, forming syncytial cysts in which each cell is connected via a single ring canal to the central cytoplasm (cytophore). Then, during meiotic prophase (in diplotene), synchrony is lost, and it is likely that one cell per cyst begins accumulating nutrients and differentiating into an oocyte. As oocytes detach from the cyst and continue oogenesis as individual cells, the remaining cells stay interconnected, do not grow, and are regarded as nurse cells. Yolk absorption is not completed in the ovary; vitellogenic oocytes are transferred to the ovisacs, where they continue to accumulate nutrients. Ovisacs are paired, long, sac-like structures, extending through several body segments (XII-

XV). *Delaya* produces mesolecithic eggs with prominent yolk spheres, lipid droplets, and glycogen granules. Only some minor differences were observed between the two studied species. The most notable difference concerns the cytophore shape and volume in cysts connecting nurse cells. In *Delaya* sp. FR, the cytophore is reticular and inconspicuous, whereas in *Delaya* sp. GR, the cytophore is more prominent and may contain nurse cell nuclei.

The obtained results confirm that the formation of the germline cysts equipped with the cytophore is a conservative phase of oogenesis in clitellates. Morphological observations suggest that in *Delaya*, the clustering cells differentiate into two subpopulations: oocyte and nurse cells, which aligns with the reports presenting oogenesis in other clitellates. Considering the differences in ovary organization between *Delaya* and other clitellates, we propose to refer to these as "Delaya-type" ovaries. The main similarities and differences between "Delaya" ovaries and other clitellate annelids are discussed. It is suggested that the presence of cysts equipped with the reticular cytophore could be an apomorphy of Pelodrilidae, earthworms, and allied taxa.

We also provide DNA barcode sequences for *Delaya* sp. FR to shed light on its taxonomic identity. Furthermore, the phylogenetic analysis that was conducted indicates that *Delaya* sp. FR occupies a basal position among its congeners for which molecular data are available.

**Keywords**: Oligochaeta, Haplotaxidae, germline cysts, oocytes, nurse cells, cytophore, ring canals, gametogenesis, DNA barcoding, Clitellata phylogeny, microdriles, megadriles.

## 1. Introduction

Clitellata is a monophyletic taxon grouping hermaphroditic annelids with a clitellum (saddle). This taxon is traditionally divided into clitellates with tiny chaetae (Oligochaeta) and leeches (Hirudinea), which lack chaetae and are predators or bloodsuckers. Oligochaeta comprises two ecological groups, i.e., the minute and mainly freshwater-associated worms loosely termed microdriles (e.g., pot worms or sludge worms) and large, terrestrial, and burrowing earthworms (loosely termed megadriles) (Erséus, 2005; Jamieson, 2006; Michaelsen, 1919; Timm and Martin, 2015). Classification of taxa within Clitellata is still under debate, and new morphological and, significantly, molecular data modify the existing systems (Anderson et al., 2017; Erséus, 2005; Erséus et al., 2020; Martin et al., 2024; Schmelz et al., 2021). One of the most critical morphological aspects of Clitellata taxonomy is the organization of male and female reproductive systems. The number and exact localization of gonads, localization of gonoducts, and genital pores are essential diagnostic features used widely in the traditional and modern taxonomy (Beddard, 1895; Erséus, 2005; Jamieson, 1988; Michaelsen, 1928; Sims, 1980; Stephenson, 1930; Timm and Martin, 2015).

The female reproductive system of clitellates usually comprises one pair of ovaries (two pairs in some taxa or rarely unpaired gonads), which typically are connected via a narrow ligament to the intersegmental septum and lie free in the segment cavity (most clitellates) or are directly enveloped by a celomic epithelium (leeches) (Gates, 1976; Świątek and Urbisz, 2019). Ovarian funnels collect growing oocytes that exit the ovaries and guide them to ovisacs (egg-sacs, a protrusion of the coelomic epithelium in the next segment posterior to the ovarian segment) that store oocytes till the time of reproduction, when oocytes via oviducts and gonopores are released into a cocoon, where fertilization occurs. Spermathecae are organs that belong to the female reproductive system and store sperm from a partner (Edwards and Arancon, 2022; Jamieson, 2006). However, the number and

localization of ovaries and other elements of the female reproductive system in given segments were, and still are, important and widely used in taxonomic and phylogenetical considerations of clitellates (Brinkhurst, 1984; Brinkhurst and Jamieson, 1971; Erséus, 2005; Jamieson, 1988; Sims, 1980) the process of oogenesis and ovary histology and ultrastructure did not attract the attention for years. In the late XIX and early XX centuries, numerous light microscopy studies on the oogenesis in dozens of representatives of microdriles, megadriles, and leeches were published (summarized and discussed in Beddard, 1895; Michaelsen, 1928; Stephenson, 1930). However, until the beginning of the XXI century, electron microscopy studies on clitellate ovaries were rare and not systematic (Świątek and Urbisz, 2019).

During the last 20 years, a publication cycle devoted to ovary micromorphology and oogenesis in microdriles and leeches has been published (reviewed in Świątek and Urbisz, 2019). Recently, our group started systematically analyzing ovaries in earthworms (Raś et al., 2025a, 2025b; Świątek et al., 2023a, 2023b). All these studies demonstrated that despite the morphological differences in ovary organization and localization, clitellate ovaries are almost always composed of germline cysts ensheathed by somatic cells (with one known exception, see Świątek and Urbisz, 2019). Germline cysts are groups of transiently interconnected germ cells, and their formation is considered a conservative and widespread phase of early gametogenesis in metazoans (Brubacher, 2024; Chaigne and Brunet, 2022; Gerhold et al., 2022; Pepling et al., 1999; Spradling, 2024; Świątek and Urbisz, 2019). In male and female germline cysts of clitellates, as a rule, each clustering cell (cystocyte) has one intercellular bridge (cytoplasmic bridge, ring canal) connecting it to the central and anuclear cytoplasmic mass, termed the cytophore (Świątek et al., 2009; Świątek and Urbisz, 2019). The male cysts in Clitellata have a balloon-like appearance with the spacious cytophore in the center and numerous germ cells at the periphery (Ferraguti, 1999; Jamieson, 2006). Despite the same pattern of organization (each cell connected via one ring canal to the cytophore), female cysts

in Clitellata show a high variety in their organization. They may differ in the number of interconnected cells and cytophore shape and volume. These seemingly minor differences lead to significant differences in the organization of the cysts and ovaries, and several types of cysts/ovaries were described among clitellates (reviewed in Świątek and Urbisz, 2019). What is essential is that the given pattern of cyst/ovary organization seems to be conserved at the family (or subfamily in the case of Naididae) level and could be potentially used in phylogenetic considerations (Świątek et al., 2012; Świątek and Urbisz, 2019; Urbisz et al., 2021).

The genus *Delaya* (Fig. 1) groups narrow and elongated (around 100-120 mm) freshwater oligochaetous clitellates (microdriles), which inhabit some European caves (Delay, 1970; Hrabě, 1963). This genus was erected by Brinkhurst (1988) and groups five species. Until recently, *Delaya* and the other seven morphologically similar genera have been classified as members of the family Haplotaxidae (Brinkhurst, 1988; Martin et al., 2008). Haplotaxids attracted zoologists' attention due to, e.g., anatomical simplicity and potential plesiomorphic characters of the reproductive system (two pairs of testes followed by two pairs of ovaries – the so-called octogonadal battery) and a worldwide but discontinuous distribution (summarized in Martin et al., 2024). Numerous morphological and molecular analyses suggested that Haplotaxidae is not a monophyletic taxon but groups several evolutionarily separated lines (reviewed in Martin et al., 2024). Indeed, the most recent morphological and molecular analyses showed that this taxon comprises at least five separate clades associated with different clitellate lines (Martin et al., 2024). According to molecular studies, Palearctic *Delaya*, together with Australasian *Pelodrilus* and *Hologynus* species, form a monophyletic taxon (family Pelodrilidae) closely related to earthworms (Martin et al., 2024). On the other hand, several morphological and histological features differentiate pelodrilids from earthworms. Some are connected with the female reproductive system (as differences in ovary

localization) and the formation of yolky (mesolecithic) eggs versus oligolecithic eggs produced in most earthworms (Martin et al., 2024). However, due to a lack of data about the ovary organization in pelodrilids, it was not possible to compare its organization with the literature data devoted to earthworms and other former haplotaxids.

The present paper aims to analyze the gross morphology, histology, and ultrastructure of *Delaya* ovaries and ovisacs and to describe the course of oogenesis. Our objective was to find and classify the crucial ovarian characteristics, e.g., gross ovary morphology and histology, and the presence of germline cysts, and compare them to characters already described in microdriles and megadriles. Such analyses would be helpful in the debate on the position of pelodrilids within Clitellata, but, first of all, deliver new data about the reproductive biology of these poorly known animals.

## 2. Materials and Methods

### *2.1 Material collecting*

*Delaya* specimens were collected in two sites: in central Greece in Melissotrypa Cave (39.8778° N, 22.0492° E) and in southern France in Grotte-évent de la Follatière or Follatière Cave (43.864297° N, 3.5263° E). The collections in Melissotrypa cave were done in May 2023 and April 2024. Six specimens were collected, and the whole worms were initially fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) in field conditions. These specimens are labeled as *Delaya* sp. GR. Specimens from Follatière Cave were collected in December 2023 and August 2024. In total, nine specimens were collected. They are labeled as *Delaya* sp. FR. Their anterior parts (first XX segments) were initially fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4), whereas the rest of the bodies were fixed in 90% ethanol. In the case of both localizations (Greece and France), after the initial fixation,

the material was transferred to the Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice.

*2.2 DNA barcoding*

In the case of *Delaya* sp. GR, no barcoding was done. These specimens were collected in the exact location as those used for the recent analysis of the taxonomic status of Haplotaxidae (Martin et al., 2024). The GenBank accession number for the COI of *Delaya* sp. GR from the latter study is PP988443. In the case of *Delaya* sp. FR, sequences from six specimens were obtained. Small pieces of the body wall were excised from ethanol-fixed body fragments. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Fragments of four commonly employed marker genes were amplified for each sample: mitochondrial cytochrome c oxidase subunit I (COI) and 12S rRNA (12S), nuclear 18S rRNA (18S), and 28S rRNA (28S). Polymerase chain reactions (PCRs) were carried out in 50 µl reactions consisting of 21 µl dd$H_20$; 25 µl of Color OptiTaq PCR Master Mix (2x) (EURx, Gdansk, Poland); 1 µl of each primer at 10 mM concentration; and 2 µl of total genomic DNA as a template. All primers and the thermal profile are listed in Table S1. PCR products were run and checked on a 1.2% agarose gel in TBE buffer with the addition of SimplySafe (EURx). Amplification products were sent to GenoMed (Warsaw, Poland) and sequenced in both directions. The resulting sequences were analyzed using BLAST (at NCBI) (Camacho et al., 2009) and Barcode ID (at BOLDSystems) (Ratnasingham et al., 2024) tools. The newly generated DNA sequences in the present study were deposited in GenBank under accession numbers: PV365189 (COI), PV365047 (12S), PV451049 (18S), and PV535595 (28S).

2.3 Phylogenetic analysis

We conducted a multi-locus phylogenetic analysis to infer the relationships between *Delaya* species used in our study and other members of Pelodrilidae. Sequences generated in this study for COI, 12S, and 18S from *Delaya* sp. FR were supplemented with sequences retrieved from GenBank for other clitellates (Supplementary Table S1), most of which had been used in a recent study by Martin et al. (2024). Nucleotide sequences were aligned independently for each locus using MAFFT v7 (Katoh et al., 2019). The alignments were trimmed to retain defined boundaries for each locus based on the available sequence data. The COI, 12S, and 18S alignments consisted of 657, 389, and 950 aligned sites, respectively. The final concatenated dataset matrix, comprising 30 terminals, consisted of 1996 aligned sites (Supplementary Data S1).

A web server version of IQ-TREE (Trifinopoulos et al., 2016) was used to conduct phylogenetic analyses and select the best-fit models of nucleotide evolution. We applied a partitioning scheme in which the optimal substitution model was determined separately for each codon position of the protein-coding COI and non-protein-coding locus. The phylogenetic tree was inferred using the models selected by IQ-TREE: TIM3e+G4, TPM3u+F+I+G4, and TN+F+G4 for the first, second, and third codon positions of COI, respectively; GTR+F+I+G4 for the 12S locus; and TNe+I+G4 for the 18S locus. Ultrafast bootstrap (UFBoot) and SH-like approximate likelihood ratio test (SH-aLRT) support values were estimated using 1000 replicates under default settings. Following previous phylogenetic hypotheses (Erséus et al., 2020), the tree was rooted at *Capilloventer australis*. The final tree was visualized using iTOL (Letunic and Bork, 2024).

*2.4 Light and transmission electron microscopy*

The initially fixed anterior parts of *Delaya* sp. GR and *Delaya* sp. FR were fixed again in laboratory conditions at room temperature with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for two to three days. Then, the ovaries and ovisacs were dissected from gonadal segments, photographed in a stereomicroscope or with differential interference contrast (see section 2.4), and then processed to obtain the resin blocks. After washing in a phosphate buffer, the ovaries and ovisacs were postfixed for 2 h in 1% $OsO_4$ in the same buffer, dehydrated in a graded series of ethanol that was replaced by acetone, and embedded in an Epoxy Embedding Medium Kit (Sigma, St. Louis, MO). Semithin sections (0.7 μm thick) were cut on an RMC Power XT ultramicrotome (RMC Boeckeler, Tucson, AZ) and stained with 1% methylene blue in a 1% sodium biborate solution at room temperature for 30 s. Next, the sections were examined using an Olympus BX60 microscope equipped with an XC50 digital camera (Olympus, Tokyo, Japan) and cellSens Standard software (Olympus, ver. 1.8.1). Ultrathin sections (50-60 nm thick) were cut on a Leica 7 ultramicrotome (Leica Microsystems, Wetzlar, Germany). The ultrathin sections were contrasted with uranyl acetate (30 min) and lead citrate (20 min). The contrasted sections were examined using a Hitachi H500 transmission electron microscope at 75 kV.

*2.5 Stereomicroscope and differential interference contrast*

For stereomicroscope analysis, the gonadal segments or isolated ovaries and ovisacs were placed on Petri dishes and analyzed with a Leica M205C stereomicroscope equipped with an Olympus ZX81 camera. Additionally, ovaries were whole-mounted onto microscope slides and observed under an Olympus BX63 microscope equipped with a Teledyne Photometrics Prime BSI camera and an Olympus cellSens Dimension software under Nomarski differential interference contrast.

## 3. Results

### *3.1 Genetic characterization and phylogenetic analysis*

We successfully amplified all four target marker genes, obtaining a 1081 bp fragment of COI, a 531 bp fragment of 12S, a 1772 bp fragment of 18S, and a 328 bp fragment of 28S. The COI gene was amplified using the universal primer pair LCO1490/HCO2198 and a combination of LCO1490 and HHCO primers to generate the longer fragment. No sequence variation was detected in COI, 12S, 18S, and 28S among the six analyzed specimens of *Delaya* sp. FR. Members of the genus *Delaya* exhibit substantial genetic divergence (ca. 15–21%) in the Folmer region of COI sequences. Notably, BLASTn analysis (search for somewhat similar sequences) of the COI Folmer region from *Delaya* sp. FR returned the highest similarity match with *Delaya* sp. IT isolate CE7161, followed by earthworms and enchytraeids. These latter two groups showed slightly higher nucleotide identity (at ≤99% query coverage) compared to other *Delaya* congeners (at 100% query coverage), which were disfavored in BLAST scoring. Earthworms and enchytraeids appeared exclusively as top matches when analyzing the longer COI fragment, for which no *Delaya* sequences of comparable length were available. In contrast, BLAST results for 12S returned *Delaya* spp. as the top hits (excluding *Delaya* sp. IT isolate CE7161), indicating that this marker is more conserved within the genus. The 18S sequence of *Delaya* sp. FR shows high similarity (99% identity) and no gaps across all currently available *Delaya* species, except *Delaya bureschi* sequence (Acc. HE800207), which exhibits lower similarity, gaps, and numerous degenerate positions in the region located downstream of the sequence corresponding to Frw921b18S primer (5'-TTCTTGGATCGCCGTAAGAC-3'; de Carle et al., 2022). Given the high conservation of 18S across other *Delaya* species, the discrepancies in *D. bureschi* (Acc. HE800207) suggest potential issues with the sequence quality/assembly in that region. No matches for *Delaya* were found in BOLDSystems based on COI and secondary markers using the Barcode ID

tool. The 18S search returned hits in members of Lumbricidae with approximately 98% similarity.

Phylogenetic reconstruction of *Delaya* species required at least three genetic markers to resolve the Pelodrilidae clade confidently and to cluster all available *Delaya* members, including *Delaya* sp. IT isolate CE7161 (which was placed outside of Pelodrilidae in the preliminary COI + 12S analysis; not shown). In the phylogenetic analysis (Fig. 2), we recovered Pelodrilidae as a well-supported clade, with *Delaya* sp. FR positioned as the most basal lineage within the *Delaya* cluster. *Delaya* sp. GR was recovered as a sister to *Delaya bureschi*, consistent with the findings of Martin et al. (2024). *Delaya* clade itself was recovered as a sister to the remaining Pelodrilidae (*Pelodrilus* plus *Hologynus*). Furthermore, Pelodrilidae was resolved as a sister to Alluroididae plus Crassiclitellata (Metagynophora), corroborating previous results (Martin et al., 2024).

### *3.2. Gross ovary and ovisac morphology*

Both species studied have two pairs of ovaries, one in the XII segment and the second in the XIII segment (Fig. 3A-B, E, 4A, 5A). Ovaries are conical, with narrow apical parts attached to intersegmental septa 11/12 and 12/13 (Fig. 3A-B, E, 4A, 5A). The more enlarged part (distal) contains growing oocytes and is suspended in the coelomic fluid of the segment (Fig. 3A-B, E, 4A, C-D, 5A-C). Ovaries are localized close to the alimentary tract and bundles of blood vessels (Fig. 3A-B, 4A, 5A).

All four ovaries in a given specimen are morphologically broadly similar (Fig. 3A-B, 4A, 5A). However, the ovary and ovisac development level differs between specimens, which can be easily seen in the case of *Delaya* sp. FR (Fig. 3A-D). The differences are connected with the level of sexual maturity of the given specimen. In some worms, only ovaries are

detected, and no vitellogenic oocytes and ovisacs occur (Fig. 3A). In the next specimen, additionally, there are singular vitellogenic oocytes within the segmental cavity (Fig. 3B). In the other specimens, the large ovisacs are filled with late vitellogenic oocytes and cover the ovaries and other organs tightly (Fig. 3C-D). In this case, ovaries can be seen after the removal of ovisacs (Fig. 3D). Thus, the ovaries have similar morphology in all specimens, but the difference is in the progress of oogenesis, which is manifested in the growing number of oocytes per ovary and their increasing volume, and the enlargement of the ovisac size.

Ovisacs have a form of more or less developed irregular sacs filled with absorbing yolk (vitellogenic) oocytes (Fig. 3C-E, 4B, 5C, D). As described, ovisac dimensions change due to the gathering of growing oocytes. The maximal size of ovisacs was observed in *Delaya* sp. FR, stretching between the first ovarian segment (XII) and XV-XVI segments (Fig. 3C-E). A rich network of blood vessels covers ovisacs (Fig. 3C-D, 4B, 5C).

Below, the histological and ultrastructural details of the ovaries and ovisac components are presented. Special attention is paid to the germline cells and oocyte development. It should be noted here that there are differences in the properties of fixed organs and cells, most probably due to different conditions of material fixation (in the case of *Delaya* sp. GR, all specimens were fixed in a field condition only – see methodology). In the case of *Delaya* sp. GR, cells slightly shrank compared to *Delaya* sp. FR. Unless indicated otherwise, the results are presented for both species studied.

### *3.3. Ovaries are composed of several units*

Each ovary is conically shaped with a narrow apical part filled tightly with numerous small cells and connected to the septum. In contrast, the distal, broad part comprises mainly growing oocytes and hangs freely in a segmental cavity (Fig. 4C-D, 5B-C, 6A-B). Microscopic analyses revealed that each ovary consists of a few (three to a maximum of

seven) units of similar morphology, i.e., with the apical narrow part and broader distal one (Fig. 4C-D, 5B-C, 6A-B). Ovarian units adhere closely and form a compact ovary (Fig. 4C-D, 5B-C). However, each unit is enveloped by its thin somatic sheath (see section 3.6); it can be easily separated from another, especially at the apical part of the ovary (Fig. 6A-B). At their distal parts, these units usually adhere more closely to one another (Fig. 4D, 5C, 6A-B). Still, they can also be separated here (Fig. 4C). All units are attached at the same site to the septum (Fig. 5B-C), and no somatic envelope ensheathing the whole ovary (i.e., ovarian envelope) has ever been found. Thus, each of the four ovaries in a given specimen comprises several closely adhering units, separated from one another by a thin somatic envelope and attached to the septum at the same site but not surrounded by a common sheath.

*3.4. Early oogenesis – ovarian unit*

The apical part (zone I) is tightly filled with a mass of small cells, and there are no growing (vitellogenic) oocytes here (Fig. 5B-C, 6A-C, 7A-C). These are mainly germline cells associated with some somatic (follicular) cells (Fig. 7A-C, 8A-E; see section 3.6). Germline cells are oogonia and germ cells that have already started the meiotic prophase I, from leptotene to diplotene (Fig. 7A-C, 8A-E). Oogonia have nuclei with dense patches of heterochromatin, prominent nucleoli, mitochondria forming small aggregations (clouds), cisternae of rough endoplasmic reticulum, and prominent Golgi complexes (Fig. 7A, C, 8A-B). Mitotically dividing oogonia occur in zone I; usually, more than one mitotic plate can be observed on a section, which suggests that oogonia interconnected in a given cyst divide, at least, in partial synchrony (Fig. 7A, C). When meiosis starts, the chromatin condenses and forms meiotic chromosomes attached to the nuclear envelope at one nuclear pole; this is the so-called bouquet stage (zygotene-pachytene) (Fig. 7A-B, 8C-D). Synaptonemal complexes can be observed in such nuclei (Fig. 8D). Meiotic cells in a bouquet stage have a similar cytoplasm ultrastructure to oogonia (Fig. 8C-D). When bivalents start to separate, no distinct

chromosomes can be observed, and chromatin in meiotic cells is dispersed; thus, the diplotene begins (Fig. 7B, 8D-E). Early diplotene cells are slightly bigger on sections; their nuclei have prominent nucleoli, and the cytoplasm has similar content as in oogonia and leptotene-zygotene cells (Fig. 7A-B, 8D-E).

Both oogonia and early meiotic cells are interconnected by stable cytoplasmic bridges (intercellular bridges or ring canals) and form syncytial cysts (Fig. 8A-E). The ring canal diameters are relatively low and range from 0.7 μm to 1.7 μm (the mean diameter is 1.13 μm, n=8). The pattern of cyst organization is the same as found in other clitellate annelids, i.e., each cell has one ring canal connecting it to the central cytoplasmic mass (Fig. 8A-E). This central cytoplasmic core is devoid of nuclei and is traditionally termed cytophore. The cytophore has a form of irregularly shaped cytoplasmic strands stretched between interconnected cells (Fig. 8A-E). The cell membrane forming the bridge is lined with a layer of electron-dense material (the so-called bridge rim) (Fig. 8A-E). The cytophore membrane is also lined by dense fibrous material, but this material is not continuous – as cytophore volume increases, this material gradually disappears (Fig. 8D-E). In foci where this material is absent, the cytophore may form thin lateral cytoplasmic projections (Fig. 8A-C). Such a cytophore formed by thin cytoplasmic strands is termed the reticular cytophore. Ring canals and cytophore contain cytoplasm with similar content as cells' cytoplasm, i.e., mitochondria, cisternae of smooth endoplasmic reticulum, ribosomes, and cytoskeletal elements, such as microtubules, can be observed here (Fig. 8A-E). However, Golgi complexes have not been observed in ring canals and cytophores. All interconnected cells in a given cyst are in the same stage of the cell cycle, i.e., cysts with clustering oogonia (Fig. 8A-B), cysts with cells in zygotene-pachytene (bouquet stage) (Fig. 8C), and cysts interconnecting early diplotene cells can be observed (Fig. 8D-E). Cysts with oogonia occupy the top of zone I; below are cysts with early meiotic cells (Fig. 7A-B).

Most of the ovarian unit volume is occupied by growing oocytes (early vitellogenic and vitellogenic, the latter occupy terminal positions within units) and nurse cells – this distal part is defined as zone II (Fig. 6A-F; 9A-E, 10A-F). Here, the synchrony of interconnected diplotene cells is lost. Each cyst breaks up into an individual oocyte, most probably no longer connected via the ring canal to the cytophore (oocyte ring canal has not been ever found on sections), and the nurse cells, which are still interconnected via ring canals to the cytophore (Fig. 10C-F, 11G, Fig. 12F). Oocytes gradually gather cell organelles and nutrients and grow considerably (Fig. 9A-E, 10A-F). A previtellogenic phase of oocyte growth (phase before yolk accumulation) seems to be relatively short; a small number of such oocytes on sections can be found (Fig. 9A, 10B). Previtellogenic oocytes are slightly bigger than nurse cells; the cytoplasm contains a relatively low number of organelles (Fig. 11A, 12A). The oolemma is smooth or tends to form microvilli (Fig. 11A, 12A). Most oocytes found in ovarian units are considered early vitellogenic (Fig. 6A-F, 9A-C, 10A, C-D). They gather cytoplasm enriched with mitochondria accumulations, Golgi complexes, and short cisternae of endoplasmic reticulum (Fig. 11B-E, 12B-C). Additionally, some minor accumulations of reserve material occur in these cells (Fig. 9A-C, 11D-E, 12C). Mitochondria accumulations found in these oocytes attract attention. In *Delaya* sp. FR, mitochondria accumulate along the cortical cytoplasm or are close to the oocyte nucleus (germinal vesicle) (Fig. 9A-C, 11C-E). Mitochondria are spherical in sections, closely adhere to one another, and lack cristae (Fig. 11C-E). In *Delaya* sp. GR elongated, thread-like mitochondria accumulate in the oocyte interior; however, some mitochondria can also be observed close to the cortical cytoplasm (Fig. 12B-C). Germinal vesicles (oocyte nuclei) contain prominent nucleoli, and sometimes, the electron-dense nuclear bodies can be observed; chromatin is dispersed and merely visible on sections; the nuclear envelope is irregular (Fig. 9A-C, 11C, 12B-C). Germinal vesicles are located eccentrically, usually close to the oocyte periphery (Fig. 6D-F, 9A-C). Vitellogenic

oocytes occupy the terminal position in ovarian units (Fig. 6B, F, 9D-E, 10E). The maximal measured diameter (in the longest axis) of the terminal oocytes was 220 µm in *Delaya* sp FR (mean = 170 µm, n=6). They have similar cytological properties to early vitellogenic oocytes, but are slightly bigger on sections and contain much more reserve material. Their cytoplasm becomes enriched in dense proteinaceous yolk granules and tiny lipid droplets (Fig. 11F, 12D-E). Lipid accumulations are especially prominent in *Delaya* sp. FR, where they form compact aggregations at one oocyte pole, easily visible even at a stereomicroscope and Nomarski contrast (Fig. 4A, C-D), and on histological sections (Fig. 9D-E). Such accumulations are not observed in the case of *Delaya* sp. GR. In both species, degenerating oocytes occur; their cytoplasm is dense, and their shapes are rounded (Fig. 9A, C).

Nurse cells are much more numerous but smaller than growing oocytes (Fig. 6A-B, D-F, 9A-E, 10A-F). In contrast to growing oocytes, they are still interconnected by ring canals to the cytophore and form cysts (Fig. 10C-F, 11G-I, 12F-G). Nurse cells in both species have nuclei with prominent nucleoli (Fig. 9A-E, 10A-F, 11G, I, 12F-G). In their cytoplasm, such organelles as mitochondria, endoplasmic reticulum, and Golgi complexes are present (Fig. 11G-I, 12F-G). They may also contain lipid droplets (Fig. 11H). Surprisingly, some important differences in cytophore organization between the studied species have been observed. In *Delaya* sp. FR, the cytophore has the form of thin cytoplasmic strands that can be recognized only at the ultrastructural level (Fig. 11G). In contrast, in *Delaya* sp. GR cytophores are more prominent. They are elongated or rounded and can be easily observed at the light microscopy level (Fig. 10C-F). In *Delaya* sp. GR cytophores are especially broad in cysts accompanying terminal oocytes (Fig. 10E-F), and they may contain even nurse cell nuclei (Fig. 10F, 12G). It is interpreted as an early phase of nurse cells and cytophores degradation. In *Delaya* sp. FR, degenerating nurse cells can also be observed. They are much denser than other nurse cells,

and their shape is strongly irregular (Fig. 9D, 11H-I). However, in *Delaya* sp. FR, prominent cytophores with embedded nuclei have never been observed.

*3.5. Late oogenesis – ovisac*

Oocytes continue to grow within the ovisacs. Here, they reach the maximal observed dimensions (Fig. 5D, 13A, C) (650µm in the longest axis), and they are interpreted as late vitellogenic oocytes (Fig. 13A-D, 14A-E). However, some smaller (vitellogenic) oocytes can also be found within ovisacs (Fig. 13A). Oocytes' shape is irregular, and ooplasm may form projections (Fig. 5D, 13A-B). Late vitellogenic oocytes still absorb yolk precursors – coated pits occur in the cortical ooplasm (Fig. 14E), and their cytoplasm is tightly filled mainly with proteinaceous yolk spheres (Fig. 13A-D, 14A-E). Numerous lipid droplets and glycogen granules are scattered among yolk spheres (Fig. 14A-E). In *Delaya* sp. FR, aggregations of mitochondria and lipids similar to those found in vitellogenic oocytes have never been found. In *Delaya sp. GR*, some aggregates of mitochondria still occur in the internal ooplasm (Fig. 14C). Oocytes in ovisacs are still in meiotic prophase I, and germinal vesicles contain prominent nucleoli (Fig. 13A, C-D). Chromatin is dispersed, and meiotic chromosomes are hard to observe in sections (Fig. 13D). The oocytes arrested in meiotic metaphase I have not been observed. Concerning cortical ooplasm, a delicate fibrous material occurs in *Delaya* sp. GR among well-developed microvilli (Fig. 14B) is interpreted as a vitelline envelope. In the biggest observed oocytes of *Delaya* sp. FR, microvilli are not so prominent, and the vitelline envelope is hard to recognize (Fig. 14D-E), which could be connected with the cessation of yolk absorption and enlargement of cell size. It is worth noting that nurse cells and cytophores have not been found within ovisacs.

Figures 15 and 16 present the schematic interpretation of the ovary, ovisac, and germline cyst organization in *Delaya*.

*3.6. Somatic components*

Germline cells found within ovaries and ovisacs are associated with somatic cells. Somatic components form the envelope of ovarian unit (Fig. 6A-B, 7A-C, 8A, 9A-C, 10B, 11H, D) and the ovisac envelope (Fig. 13A-B). Somatic cells also connect ovarian units to the septum (Fig. 5B, 6A, 7A). As was mentioned, no ovarian envelope (i.e., an envelope ensheathing all units together) was found (Fig. 4C-D, 5B-C). Follicular cells directly adhere to germline cysts and growing oocytes (Fig. 7A-C, 8A-E, 9C-D, 10C-F, 11B, D-E, H, 12A-B, D, F-G). Somatic cells, both forming envelopes of ovarian units and follicular cells, are elongated and form thin cytoplasmic projections (Fig. 7A-C, 8A-E, 9C-D, 10C-F, 11B, D-E, H, 12A-B, D, F-G). In the case of follicular cells, these projections intermingle and form a network around growing oocytes, nurse cells, and cytophores (Fig. 9C-D, 10C-F, 11B, D-E, H, 12A-B, D, F-G). Cell junctions recognized as desmosomes interconnect projections of follicular and envelope cells (Fig. 8A, C, 11H). Follicular cells enveloping oocytes in ovisacs have not been observed. The ovisac wall also contains muscle fibers (not shown) and is enriched in blood vessels (Fig. 4B, 5B, 13A-B).



## 4. Discussion

### 4.1. Selected aspects of oogenesis

*4.1.1. Germline cysts*

It is widely accepted that the formation of syncytial groups of germ cells, where each cell has at least one ring canal connecting it directly or indirectly with other clustering cells, is an evolutionarily conserved aspect of animal gametogenesis (Brubacher, 2024; Chaigne and Brunet, 2022; Lu et al., 2017; Pepling et al., 1999; Spradling, 2024; Spradling et al., 2022). The formation of cysts during spermatogenesis is ubiquitous, and spermatogonia,

spermatocytes, and spermatids develop as interconnected cells in almost all animals (Chaigne and Brunet, 2022; Roosen-Runge, 1977; Yoshida, 2016). In contrast, the situation is much more complicated in the female germline. Female cysts were never observed in some taxa (such as mollusks or echinoderms) (Brubacher, 2024; Świątek and Urbisz, 2019). In numerous insects, including *Drosophila melanogaster*, some crustaceans, tardigrades, nematodes, and clitellate annelids, oogonia, nurse cells, and oocytes stay interconnected until advanced oogenesis (Büning, 1994; Jaglarz and Bilinski, 2020; Poprawa and Janelt, 2019; Seidel et al., 2018; Świątek and Urbisz, 2019). In some insects (e.g., stone flies) and numerous vertebrates (as some fish, frogs, and mammals), only oogonia are interconnected, and cysts break down when meiosis begins (Gottanka and Büning, 1990; Kloc et al., 2004; Marlow and Mullins, 2008; Spradling et al., 2022). The cyst formation and functioning are well-known in such model species as *D. melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, *Xenopus laevis*, and *Mus musculus* (Bilinski et al., 2017; Kloc et al., 2004; Kumar and Elkouby, 2023; Seidel et al., 2018; Spradling et al., 2022). Our molecular knowledge about cyst origin and functioning is poor or completely lacking in other animals, including clitellate annelids. Generally, it is known that in the case of spermatogenesis, the interconnection of cells is responsible for the synchronization of sperm development and sharing of gene products between haploid spermatids (Guo and Zheng, 2004; Ventelä, 2006). The interconnected cells (cystocytes) usually diversify their fates during oogenesis, and cysts are polarized. Only a small population of cystocytes (sometimes one) in a given cyst becomes an oocyte, while the rest play the role of auxiliary cells (known as nurse cells or trophocytes) that supply oocytes with macromolecules (e.g., mRNA) and organelles (e.g., mitochondria). This so-called “nourishing mechanism” is widely considered a strategy that accelerates oogenesis (Brubacher, 2024; Chaigne and Brunet, 2022; Lei and Spradling, 2016; Lu et al., 2017; Pepling and Spradling, 2001). Ovaries, where oocytes and nurse cells emerge, are termed as

meroistic, and it is believed that in such cases, there is unidirectional transfer of macromolecules and organelles from nurse cells toward oocyte(s). When cysts are absent or break down very early (before meiosis), and all formerly interconnected cystocytes continue meiosis as individual cells, the panoistic meiosis occurs (Biliński, 1998; Brubacher, 2024; Świątek and Urbisz, 2019).

Our ultrastructural analyses have revealed that gemline cysts in both studied *Delaya* species are formed during early oogenesis. It is known from studies on model organisms that cysts are formed by incomplete cell divisions, where there is no complete separation of cells during late cytokinesis, and the contractile ring is transformed into the ring canal (Chaigne and Brunet, 2022; Gerhold et al., 2022; Greenbaum et al., 2011; Haglund et al., 2011). Our observations of cyst formation were limited to noticing that in *Delaya,* at least some oogonia divide in synchrony. Most probably, two or more oogonia forming a given cyst divide synchronously. However, we have no direct and quantitative data to support our suspicion based on morphological observations that all oogonia in a given cyst divide in synchrony. It is well-known that the inerconnection of cells into a cyst is connected with the synchronization of the cell cycle (Cuevas et al., 1997; Huynh, 2006; Yamashita, 2018). Such synchrony results in the formation of cysts with a stable, and usually taxon-specific, number of cells. The number of interconnected cells is usually fixed and defined by the $2^n$ rule, where n = means the number of synchronous divisions. For instance, in *D. melanogaster* female cysts, there are 16 interconnected cells, whereas in butterflies, female cysts are composed of eight cells (Biliński, 1998; De Cuevas et al., 1997; Telfer, 1975).

In clitellates, synchronic divisions of germ cells were reported in both male and female cysts (e.g., Małota and Świątek, 2016; Świątek et al., 2009). Histological and ultrastructural observations suggest that in *Delaya,* cells interconnected in a given cyst develop synchronously until diplotene; however, we do not know how many synchronic divisions

occur and how many cells are interconnected (see below). In a given cyst, all interlinked cells have the same morphology and are in the same cycle phase. The synchrony is lost in diplotene, where two cell categories emerge – developing oocyte or oocytes and nurse cells. However, there is no complete developmental synchrony between cysts. The tip of the ovarian unit is occupied by cysts interliking oogonia, below are cysts with cells entering meiotic prophase I, then cysts with diplotene cells – all these tightly packed cysts form zone I of the units. In zone II, cysts comprise only nurse cells, whereas most of this area is occupied by growing oocytes, which develop as individual cells. The ring canals interconnecting oocytes with the cytophore have never been observed. Such observations align with numerous reports presenting the microorganization of ovaries in other clitellates, both microdriles and megadriles (reviewed in Świątek and Urbisz, 2019).

The germline cysts found in *Delaya* ovaries are equipped with the central anuclear cytoplasmic core, the cytophore. In this system, cells are not directly connected via ring canals, but each has a single bridge linking it to the shared cytoplasm (Fig. 2, Fig. 16A-D). This is one of the several systems of cyst architecture in the gonads known in Metazoa (see e.g., Brubacher, 2024; Chaigne and Brunet, 2022; Gerhold et al., 2022; Świątek and Urbisz, 2019 for more details about different systems of cyst architecture). Cysts with a central common cytoplasm, aside from annelids, have been described, e.g., in nematodes (Seidel et al., 2018; Zellag et al., 2025) or oribatid mites (Liana and Witaliński, 2012). In annelids, cysts with the cytophore are typical for spermatogenesis in all taxa (Jamieson, 2006; Olive, 1983; Rouse, 2006) and for oogenesis in spoon worms (Echiura) (Leutert, 1974) and clitellates (Świątek et al., 2009; Świątek and Urbisz, 2019). The presence of the cysts with cytophore in the studied *Delaya* species is the next evidence that the formation of cysts with this specific geometry is a conservative aspect of oogenesis in Clitellata. Such cysts have been found in the ovaries of almost all clitellates examined so far using electron microscopy. The only known

exception is *Capilloventer australis*, a representative of the basal lineage of Clitellata, where the cysts were not found, and female germ cells develop individually (Fig. 2A, Świątek et al., 2016). It should be noted that studies based on light microscopy are usually not sufficient to detect the cysts' presence, especially in cases when the cytophore is poorly developed (see below). For this reason, for a long time it was believed that the ovaries of oligochaets are panoistic, without formation of germline cysts (Jamieson, 2006, 1981).

Despite the common and conservative pattern of cysts' architecture (each cell has one ring canal connecting it to the cytophore), the detailed cyst composition, shape, and dimensions vary between clitellate taxa (Fig. 2). The observed differences are mainly related to the number of interconnected cells and the shape and size of the cytophore. Regarding the number of interlinked cells, two categories may be recognized: oligocellular (from several to dozens of interconnected cells) and multicellular cysts (hundreds or more clustering cells). Regarding the cytophore shape and volume, in the case of multicellular cysts, the cytophore is voluminous and forms broad cytoplasmic stands (tree-like cytophore) as was described in e.g., a sludge worm, *Tubifex tubifex* (Fig. 2C, Urbisz et al., 2015). The ball-like cytophore is characteristic of oligocellular cysts with dozens of cells (16-32 or around 60) and was observed in cysts in fish leeches (Spałek-Wołczyńska et al., 2008) and such microdriles as the white worm, *Enchytraeus albidus* (Fig. 2B, Urbisz et al., 2017). In contrast, the reticular cytophore, composed of thin cytoplasmic strands forming lateral projections, occurs in oligocellular cysts with only a few cells (around eight), which is typical for earthworms (Fig. 2F, Raś et al., 2025a, 2025b; Świątek et al., 2023b, 2023a).

Our ultrastructural analyses revealed that, in both studied species, the cysts have a similar ultrastructure to cysts with the reticular cytophore found in earthworm ovaries (Fig. 2F, H, Fig. 16A; Raś et al., 2025a, 2005b; Siekierska, 2003; Świątek et al., 2023a, 2023b). The reticular cytophore consists of thin cytoplasmic strands with lateral projections; what is

more, the bridge rim lines the cytophore membrane, but in places where the rim is absent, the lateral projections are formed (Fig. 16A). No prominent tree- or ball-like cytophores characteristic of microdriles have been found in *Delaya* ovaries. Thus, cysts with reticular cytophore found in *Delaya* ovarian units are similar to those known from earthworm ovaries, where eight cells per cyst were reported, which suggests three mitotic rounds of cystocyte divisions (Świątek et al., 2023a). In *Delaya*, we have never found more than three cells directly connected to the cytophore (Fig. 8D). Thus, the presence of the reticular cytophore and ultrastructural observations suggest that *Delaya* cysts are also oligocellular with a low number of clustering cells (below 10). However, we have no exact data on how many cells per cyst occur in *Delaya* due to unclear cyst limits and their complex shape. Only additional studies using ultrathin serial sectioning will clarify this issue.

On the other hand, the observations of *Delaya* cysts organization in zone II complicate this more or less clear picture. In *Delaya* sp. FR, the cytophore is still almost undetectable and poorly developed (Fig. 16C), similar to earthworm ovaries zone III (Świątek et al., 2023a, 2023b). In contrast, in *Delaya* sp. GR, the cytophores are more prominent and may contain several nurse cell nuclei (see below, Fig. 16D). This feature is not observed in earthworms but is more typical for multicellular cysts found in microdriles and leeches (Świątek and Urbisz, 2019; Urbisz et al., 2015). It remains unexplained why, in two species of *Delaya,* such remarkable differences in late cyst organization occur. Such differences could probably be connected with different scenarios of cyst degradation. The presence of nuclei inside the cytophore is interpreted as a sign of cyst degradation (Świątek and Urbisz, 2019; Urbisz et al., 2020, 2014). It seems that the mechanisms that prevent migration of the nurse cells nuclei, such as the narrow diameter of ring canals or the proper organization of the cytoskeleton, do not work fully in “old” cysts ready to be eliminated (Świątek and Urbisz, 2019). It is not known how exactly cyst elimination occurs in *Delaya*. Morphological data suggest that some

gem cells degenerate within the ovarian units via apoptosis, but these are mainly growing oocytes (Fig. 15C). Nurse cells in both species are most probably eliminated by an unknown mechanism at the distal ovary end, or they detach from the ovaries at the same time as vitellogenic oocytes and are phagocytosed in the coelom. The details of the process leading to the final fate of nurse cells in Clitellata are poorly understood; dedicated studies are lacking.

The different cyst morphology could also be species-specific, and may be explained by long-term evolution (the genetic distance in the COI gene between *Delaya* sp. GR and FR is 19%). Or, in the case of *Delaya* sp. FR, such prominent cytophores are also present, but were omitted? It should be underlined that in both studied species, the ovary organization and the course of oogenesis are very similar, with only minor differences, e.g., the formation of prominent lipid accumulations in vitellogenic oocytes in *Delaya* sp. FR, which were not found in *Delaya* sp. GR. It could not be excluded that such differences can also be connected with the different advancement of the oogenesis between specimens or/and their nutritional status. Only additional analyses, including more specimens and species, could shed more light on this issue.



*4.1.2. Character of oogenesis*

Ovaries of Clitellata, due to the presence of polarized germline cysts with oocytes and nurse cells, are considered meroistic (Świątek and Urbisz, 2019). Thus, there is believed to be a directional transfer of cytoplasm with cell organelles and macromolecules on the axis: nurse cell-cytophore-oocyte(s). Such a conclusion is based on morphological observations, where in a given cyst, one or more cells continue meiosis, develop microvilli, and gather a large amount of nutrients. In contrast, the rest of the cells (nurse cells) do not grow, do not form microvilli, and seem to be eliminated by the cell death process (Świątek and Urbisz, 2019). It

should be noted that in Clitellata, there is a lack of molecular analyses to prove directly such a "nourishing mechanism". The only experimental proof comes from studies on the leech *Glossiphonia complanata*, where incorporation of 3H-uridine into nuclei of nurse cells and then the transfer of labeled RNA into growing oocytes was demonstrated (Aisenstadt et al., 1967). In *Delaya*, the cytophore is poorly developed; moreover, growing oocytes were never found to be connected to the cytophore. This suggests that the cytoplasmic transfer is structurally (small volume of cytophore, relatively small diameter of ring canals) and temporarily (till diplotene) limited. We cannot exclude such a transfer, but it seems that in *Delaya* it has no high significance for the oogenesis acceleration. The same conclusions were drawn in the case of cysts with reticular cytophore observed in earthworms, where there are the same limitations for the cytoplasm transfer (Raś et al., 2025a, 2025b; Świątek et al., 2023a).

*4.1.3 Yolky eggs and ovisac*

Both species of *Delaya* produce huge oocytes (more than 600 µm in the longest axis) enriched in spheres of proteinaceous yolk, numerous glycogen granules, and lipid droplets, which could be classified as mesolecithic eggs. Such eggs are typical for microdriles, where primary oocytes range from 300 µm to 1 mm (Jamieson, 2006, 1981; Jamieson et al., 2002; Omodeo, 2000). In megadriles, there are two alternatives. In Crassiclitellata (true earthworms with a multilayered clitellum), eggs are small (from 20-30 µm to 100-120 µm) and contain less yolk (oligolecithic eggs), and embryos are fed by a mass of albumen secreted during cocoon formation (Jamieson, 2006; Omodeo, 2000). In Moniligastridae, a family closely related to Crassiclitellata (Anderson et al., 2017; Erséus et al., 2020; James and Davidson, 2012), the clitellum is one cell thick, as in other non-crassiclitellates (Misirlioğlu et al., 2023; Stephenson, 1930), and eggs are rich in the yolk (mesolecithic) (Beddard, 1895; Rao, 1921;

Urbisz et al., unpublished data). Aluroididae, which shows a mixture of microdrili and megadrili features, produces yolky eggs (Jamieson and Fragoso, 2024). Thus, *Delaya*, similarly to microdriles and non-crassiclitellate earthworms (i.e., Moniligastridae), gathers a vast amount of nutrients in the form of yolk.

In both *Delaya* species, oogenesis is not finalized within the ovaries. Oocytes that accumulate yolk (vitellogenic oocytes) which are, as in other animals (Matova and Cooley, 2001), in diplotene of Ist meiotic division (primary oocytes), detach from the ovary and localize in ovisacs, where they intensively absorb the yolk precursors and grow considerably (Fig. 15C). Thus, oogenesis is not completely intraovarian, and the oocyte growth is finalized in well-developed ovisacs. The terms "extraovarian" and "intraovarian" oogenesis were proposed initially by Eckelbarger to describe one of the features of oogenesis in Polychaeta (Eckelbarger, 1983). Extraovarian oogenesis means that germ cells leave the ovary before they start vitellogenesis and complete oogenesis in the coelom. In contrast, in intraovarian oogenesis, germ cells remain within the ovary during most of vitellogenesis. In literature, it is suggested that oogenesis in oligochaetous clitellates is intraovarian. After completing yolk absorption, primary oocytes arrested in metaphase I of meiosis are released from the ovaries into the ovisac or coelom (Jamieson, 2006). This does not align with the presented results and other literature data. In microdriles, oocytes rather do not complete oogenesis within the ovaries; oocytes grow in the coelom cavity or within the ovisacs (Gorgoń et al., 2017; Świątek and Urbisz, 2019). In crassiclitellate earthworms, oocytes are retained in ovaries till they gather nutrients, but it seems that in most of the studied species, oogenesis is not finished here, because the oocytes arrested in metaphase I were not observed within ovaries (Raś et al., 2025a, 2025b; Świątek et al., 2023a). Only in some earthworms from the family Megascolecidae, oocytes arrested in meiotic metaphase I have been found within gonads (Świątek et al., 2023b).

In *Delaya*, oocytes that detach from ovarian units are around 200 µm in diameter and are in prophase I of meiosis. In ovisacs, they triple their diameters and are still in diplotene. We have neither found oocytes with signs of germinal vesicle breakdown nor observed oocytes arrested in metaphase I of meiosis. However, it could be expected that in *Delaya*, when vitellogenesis is finished, oocytes restart meiosis, complete prophase I, and stop meiosis in metaphase I. In clitellates, primary oocytes in metaphase I are deposited into a cocoon when fertilization occurs, and after this, meiosis is completed (reviewed in Jamieson, 2006, 1981).

Studied *Delaya* species have well-developed ovisacs, which grow considerably when filled with oocytes. They may reach large sizes and stretch over four segments. Generally, ovisacs are pouches of the epithelium in the posterior septum of the ovarian segment, where oocytes are gathered until they are ready to lay into a cocoon (Beddard, 1895; Stephenson, 1930). In microdriles, ovisacs may be present or not – e.g., they are absent in most enchytraeids but occur in most naidins (Stephenson, 1930). In megadriles, they are absent or small in most of the families of crassiclitellate earthworms. However, they are prominent in Moniligastridae (Nguyen et al., 2022; Stephenson, 1930; Zhang et al., 2021) and occur in many representatives of Alluroididae (Jamieson and Fragoso, 2024). As mentioned, moniligastrids and alluroidids, similar to *Delaya*, produce yolky eggs; in both groups, ovisacs are well-developed (see Tab.1).

### 4.2 “Delaya” type of ovaries

As the Introduction mentions, the localization and organization of reproductive systems in Clitellata, including the gonads, is an essential taxonomic and diagnostic feature. The

localization, number, and gross morphology of ovaries could potentially be good characters to consider when discussing the taxonomic position of *Delaya.*

In both analyzed *Delaya* species, there are two pairs of female gonads in the two following segments, XII and XIII. Such ovary distribution is typical for annelids formerly grouped into Haplotaxidae *sensu lato* and was also regarded as the ancestral for all Clitellates (Brinkhurst, 1988, 1984, 1982; Brinkhurst and Jamieson, 1971). During evolution, the position of the ovaries shifted in some groups, and the number of ovaries is usually limited to one pair (Timm and Martin, 2015). Among earthworms, one pair of ovaries in segment XIII is regarded as a typical state (Edwards and Arancon, 2022; Stephenson, 1930). In *Drawida* (Moniligastridae), a pair of ovaries is located in segment XI (Gates, 1974; Stephenson, 1930). In microdriles, typically, one pair of ovaries is reported, but their localization is not stable; it varies depending on the taxon, e.g., in the VI, XI, XII, or XIII segments (Erséus, 2005; Timm and Martin, 2015). On the other hand, in Alluroididae – a family regarded as transitional between 'microdriles' and 'megadriles' – one pair of ovaries is in segment XIII, which is a 'typical' condition for crassiclitellate earthworms (Jamieson and Fragoso, 2024). Thus, despite genitalia being considered exceedingly conservative, it seems that gonad localization during the evolution of different clitellate taxa has changed independently several times. To summarize these tendencies, we listed the ovary localization in *Delaya*, crassiclitellate earthworms, and allied taxa in Tab. 1.

Regarding the organization of ovaries in *Delaya*, it is in line with the trend observed in Clitellata – ovaries show high plasticity in their external and internal organization, which differs considerably between taxa but is conserved at the family/subfamily level (Fig. 2; Świątek and Urbisz, 2019). Ovaries of *Delaya* show some specific features and do not fall into any type described to date in microdriles or megadriles. In our opinion, the organization of *Delaya* ovaries should be classified into a separate type – "Delaya" ovaries – for the

following reasons (Fig. 15-16; Tab. 1): 1) each ovary is composed of several similar and functional units; 2) the whole ovary, as well as ovarian units, are polarized – their narrow ends (apical parts) are attached to the septum and contain oogonia and early meiotic germ cells, the distal parts are broader and suspended in the segment cavity, they contain growing oocytes and nurse cells; 3) ovarian units closely adhere one to another, but each one is enveloped by a thin somatic sheath (ovarian unit envelope), there is no common sheath covering the whole ovary; 4) ovaries are composed of germline cysts (cells interconnected via ring canals and cytophore) and somatic cells; 5) in oogonial and early meiotic cysts all interconnected cells develop in synchrony, then – in diplotene – synchrony is lost and, most probably, one cell per cyst detach from cytophore and becomes an oocyte; the rest of cells (nurse cells) remain interlinked and do not continue oogenesis; 6) mesolecithic oocytes are produced, they start gathering nutrients within the ovary, but vitellogenic oocytes ovulate and the last phase of yolk accumulation occurs in well-developed ovisacs.

This description is a mixture of features conserved for clitellates (e.g., the presence of germline cysts equipped with the cytophore and cells morphologically differentiated into oocytes and nurse cells) with evolutionary novelties, such as the occurrence of the reticular cytophore and ovarian units (see Tab. 1). The phenomenon of multiplication of ovarian units is well-known in animals; e.g., insect ovaries are composed of several to hundreds of functional units termed ovarioles. In each ovariole, the oogenesis proceeds from the gonial cells till fully formed oocytes ready for fertilization (Biliński, 1998; Büning, 1994). Among Clitellata, the multiplication of ovarian units has been reported in numerous leeches and microdriles (see below). These ovarian units (known as ovarian bodies in Erpobdelidformes, ovarian cords in Hirudiniformes, and egg follicles in Piscicolidae) have different shapes and numbers but are always composed of germline cysts enveloped by somatic cells (for details, see Gorgoń and Świątek, 2021; Świątek and Urbisz, 2019).

In megadriles studied to date, ovaries are usually organs that are not divided into identical, repeated units. Such ovaries were found, e.g., in Lumbricidae and Hormogastridae (the so-called "Dendrobena" type of ovary), and are probably typical for other allied taxons (Fig. 2F) (Raś et al., 2025a; Świątek et al., 2023a). The multiplication of ovarian components found in earthworms concerns egg strings. An egg string is a row of linearly arranged growing oocytes (Gates, 1976; Świątek et al., 2023a, 2023b). In some earthworms, as representatives of the Megascolecidae, each ovary may contain dozens of egg strings (Gates, 1976; Świątek et al., 2023b). The main difference between ovarian units in *Delaya* and the egg strings found in earthworms is that egg strings contain only growing oocytes and radiate from the common core – the apical part of the ovary where oogonia and early meiotic cells occur (Świątek et al., 2023b). Thus, egg strings are not functional, independent ovarian units. It should be added here that egg strings are absent in some megadriles, and ovaries have other organization, e.g., ribbon-like ovaries in Moniligastridae (Gates, 1976; Urbisz et al., unpublished results).

In microdriles, ovaries are also not homogenous regarding their shape and internal organization; to date, five types have been described (summarized in Świątek and Urbisz, 2019; two of them are shown in Fig. 2B-D). In microdriles, ovaries are also conically shaped, with a narrow apical part attached to the spetum and a broad part containing developing oocytes and suspended in coelomic fluid (Świątek and Urbisz, 2019). In microdrile ovaries, the multiplication of some ovarian elements may also occur. These multiple elements are germline cysts, which could be clearly separated by somatic cells or float freely in the coelomic fluid. In Enchytraeidae, there are several such cysts in a given ovary, and ovaries morphologically resemble a bunch of grapes (the so-called "Enchytraeus" type of ovary; Fig. 2B, Paschma, 1962; Świątek et al., 2018; Urbisz et al., 2025, 2017). In other variations of microdrile ovaries, such as "Stylaria" (Naidinae) and "Insulodrilus" (Phreodrilidae) types,

ovaries are inconspicuous, and multiple germline cysts detach quickly from the gonad and continue oogenesis in the coelom (Gorgoń et al., 2017; Świątek et al., 2020). The main difference between the separated germline cysts in microdriles and ovarian units in *Delaya* is that in ovarian units, there are numerous cysts that, together with tightly adhering somatic (follicular cells), form a compact unit. It should be noted here that in the most widespread type of microdrile ovaries, e.g., "Tubifex" type, the whole ovary is composed of one huge germline cyst, and repeating units were not observed (Tab. 1; Fig. 2C, Urbisz et al., 2015, 2010).

The germline cysts in *Delaya* are oligocellular and have the reticular cytophore, the same pattern as found in creassiclitellate earthworms (Raś et al., 2025a, 2025b; Świątek et al., 2023b, 2023a). The same feature has been observed recently in two *Drawida* species, representatives of the family Moniligastridae (Urbisz et al., unpublished results). Moniligastrids are closely related to Crassiclitellata (Erséus et al., 2020), and it is tempting to speculate that the cysts with the reticular cytophore could be the potential apomorphy for earthworms and allied taxa. To support this idea, detailed analyses of the ovaries are needed in the next group closely related to earthworms, the Alluroididae family (Fig. 2).

The last thing worth noting is the substantial difference in the organization of the ovaries in the studied *Delaya* species and *Haplotaxis* sp., the only representative of former haplotaxids *sensu lato* where ovaries were studied (Urbisz et al., 2021). In *Haplotaxis* sp., two pairs of ovaries are located in the anterior parts of segments XI and XII, whereas in *Delaya*, they are located in segments XII and XIII. In *Haplotaxis*, each ovary is formed from one huge, multicellular cyst with a tree-like cytophore, the "Tubifex" type (Fig. 2D, Urbisz et al., 2021). *Haplotaxis* sp. also produces oocytes rich in the yolk, but they were observed in the coelomic cavity rather than in ovisacs (Urbisz et al., 2021). The occurrence of the "Tubifex" type of ovary in *Haplotaxis* sp. and the shift of gonads to XI and XII segments in contrast to

the "Delaya" ovaries and their localization in segments XII and XIII in the two studied species supports the recent molecular findings and the division of former Haplotaxidae *sensu lato* into new families (Martin et al., 2024). Additionally, regarding ovary organization, *Haplotaxis* sp. with "Tubifex" type ovaries is much closer to microdriles than megadriles. On the other hand, *Delaya* has its own scheme of ovary organization, which differs from other systems previously described in clitellates.

## 5. Summary

*Delaya* ovaries are built from germline cysts associated with somatic cells.

There are two pairs of ovaries, each composed of several identical subunits.

Cysts are equipped with the central cytophore; such a cyst pattern is the conservative aspect of oogenesis in Clitellata.

Cyst organization (reticular cytophore, small number of interconnected cells) resembles germline cysts typical for crassiclitellate earthworms. Such cyst organization could be a potential apomorphy for earthworms and allied taxa.

Due to the specific morphological organization, which is different from that known for other Clitellata, we propose the term "Delaya" ovaries.

Oogenesis is not finished in the ovaries; yolk absorption continues in well-developed ovisacs.

Yolky eggs are produced, which is typical for microdriles but not crassiclitellate earthworms.

Ovaries found in both *Delaya* species remarkably differ from those described in *Haplotaxis* sp., which supports the recent revision of the family Haplotaxidae.

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**Legends to figures**

Fig. 1. A) *Delaya* sp. GR in its natural habitat in Melissotrypa Cave. B) Three *Delaya sp. GR* specimens just after collecting. Note the different worm dimensions. C) *Delaya* sp. FR in Follatière Cave. Photos A) and B) by Serban M. Sarbu, photo C) by Bernard Galibert.

Fig. 2. The best-scoring maximum likelihood tree resulting from the analysis of *Delaya* spp. and other clitellate sequences and diagrams presenting variability in ovary organization and germline cyst architecture. Tree is based on a concatenated dataset (COI, 12S, and 18S). SH-aLRT and UFBoot support values are shown near the respective branches. Magenta squares indicate branches with both support values ≥ 80 at the respective nodes.

A and A') *Capilloventer australis* (Capilloventridae) – no germline cysts were detected in ovaries, and oocytes developed as a chain of individual cells. "Capilloventer" type of ovary. B and B') *Enchytraeus albidus* (Enchytraeidae). "Enchytraeus" ovary composed of dozens of 16-celled cysts with a ball-like cytophore (B"). C and C') *Tubifex tubifex* (Tubificinae). "Tubifex" ovary is composed of one huge multicellular cyst (around 2,000 cells) with a tree-like cytophore (C"). D and D') *Haplotaxis* sp. BEN (Haplotaxidae s. str.), "Tubifex ovaries" with tree-like cytophore (D"). E) Alluroididae – ovary organization unknown. F and F') *Apporectodea caliginosa* (Lubmricidae). "Dendrobaena" ovary with numerous olligocellular cysts with reticular cytophore (F"). G) *Pelodrilus* and *Hologynus* (Pelodrilidae). Ovary organization unknown. H and H') *Delaya* sp. FR and *Delaya* sp. GR – ovaries with oligocellular cysts equipped with reticular cytophore (*Delaya* sp. FR – H") or reticular and ball-like cytophore (*Delaya* sp. GR – H" and H'"). For more details, see the text and Figs. 15 and 16. In diagrams, somatic cells were omitted for clarity.

Fig. 3. General morphology of ovaries and ovisacs in *Delaya* sp. FR visualized by a stereomicroscope, and the scheme of gonad and ovisac localization. A) Specimen with no

ovisac. Two pairs of ovaries (double arrows) are visible. B) In the specimen with no ovisacs visible, three vitellogenic oocytes (vo) are located in the segmental cavity. Only one ovary (double arrows) on the left side is visible; ovaries on the right (red double arrows) have moved from their original position during dissection. C-D) Specimens with advanced oogenesis and prominent ovisacs filled with vitellogenic oocytes. In D, ovisacs are slightly moved to show the ovary (double arrows). Roman numerals mark the segment number; at – alimentary tract; bv – blood vessel; sv – seminal vesicle. E) Scheme depicting the localization of testes (t), ovaries (ov), and ovisacs in body segments (Roman numerals). Lateral view, thus one gonad and ovisac is presented. In fact, they are in pairs.

Fig. 4. *Delaya* sp. FR. A) General morphology of ovaries (ov) *in situ* visualized by a stereomicroscope. In early vitellogenic oocytes, lipid accumulations of milky color are well visible (stars). Short double arrows mark septa. B) Isolated ovisac visualized by a stereomicroscope. Note the rich network of blood vessels (bv). C-D) Isolated ovaries visualized by a stereomicroscope in C and by Nomarski difference contrast in D. The apical part of ovaries is marked by long arrows. The rest of the ovary comprises mainly growing oocytes with prominent lipid accumulations (black and white stars). Roman numerals mark the segment number; at – alimentary tract; bv – blood vessel.

Fig. 5. General morphology of ovaries and ovisacs in *Delaya* sp. GR visualized by a stereomicroscope. A) Lateral view of ovaries attached to the septa (double arrows) near the body wall (bw). The exact site of the attachment is not visible. The ovary in segment XII is directed upwards. B) Isolated ovary and ovisac from the same specimen. A short white arrow marks the site of the ovary attachment to the septum. C) Isolated ovary attached to the septum (double white arrows). Long white arrows mark the apical part of the ovary, in the distal part developing early vitellogenic oocytes (evo), vo – big vitellogenic oocyte. D) Late vitellogenic

oocytes (lvo) isolated from ovisac. Roman numerals mark the segment number, at – alimentary tract; bv – blood vessel.

Fig. 6. General histology and morphology of ovarian units in *Delaya* sp. FR (A, C-F) and *Delaya* sp. GR (B). A-B) Longitudinal sections; two ovarian units are visible in each case — the dashed line in A marks the border between them. Zone I (the apical part) contains a mass of small cells; no growing oocytes can be seen here. Zone II (the distal part) comprises growing early vitellogenic oocytes (evo) and vitellogenic oocytes (vo). Additionally, numerous smaller germ cells, regarded as nurse cells (nc), occur here. Note the attachment site (long arrow) of one unit to the septum (sp) and a thin unit sheath (double short arrows). Light microscopy (LM), epon semi-thin sections stained with methylene blue. C-F) The surface of the ovarian units visualized in Nomarski differential interference contrast. The abbreviations are the same as in A and B. Short arrows point to the oocyte nucleus; white stars mark the dense accumulation of lipids.

Fig. 7. Zone I histology of *Delaya* sp. FR (A-B) and *Delaya* sp. GR (C) – LM longitudinal sections. Oogonia (oo), meiotic cells in a bouquet (bu), and early diplotene (ed) stages can be seen. Note metaphase plates of dividing oogonia (short black arrows in C). A long dashed arrow in A marks the site of ovarian unit attachment to the septum (sp). Long arrows in B mark sites of chromosomes' attachment to the nuclear envelope (bouquets). Double back arrows point to ovarian unit sheath, a dashed line in B marks the border between meiotic cells in a bouquet stage and early diplotene cells, fc – somatic follicular cells scattered among germline cells (only cytoplasm is visible). Epon semi-thin sections stained with methylene blue.

Fig. 8. Ultrastructural details of germline cysts organization in zone I of *Delaya* sp. FR using transmission electron microscopy (TEM). A) Oogonia (oo). In one cell, the ring canal is visible (red ellipse); it connects the cell with a cytophore (cy). Red arrows mark the dense

rim, and black arrows – the lateral cytoplasmic projection of the cytophore. B) Higher magnification of the cyst fragment. Two interconnected oogonia (oo) are visible. C) Bouquet cells (bu) interconnected in a cyst. The cyst center is occupied by the reticular cytophore (cy); each cell has one ring canal (red ellipse). Five ring canals are visible (red ellipses) – three are continuous with cytophore. D-E). Early diplotene cells (ed) interconnected via ring canals (red ellipses) and elongated cytophore (cy). Note the discontinuous character of electron-dense material lining the bridge and cytophore membrane (red arrows). In D, the synaptonemal complexes in bouquet cells are visible (double arrows). bu – bouquet cell; ch – bouquet chromosomes; cy – cytophore; ed – early diplotene cell; er – endoplasmic reticulum; fc – follicular cells; G – Golgi complexes; m – mitochondria; nu – nucleus; oe – ovarian units envelope; oo – oogonium. Black arrows – lateral projection of the cytophore; red arrows – electron-dense material; black ellipse – desmosome-like junction; red ellipse – ring canal; white star – nucleolus.

Fig. 9. Zone II in *Delaya* sp. FR – histology visualized by longitudinal semi-thin sections. A-C) Numerous growing oocytes are visible. Previtellogenic oocytes (long arrows) are slightly bigger than nurse cells (nc). Early vitellogenic oocytes (evo) gather cytoplasm and some nutrients (red long arrows) and are much bigger than nurse cells (nc). Note accumulations of mitochondria (short red arrows) in the cortical ooplasm. Degenerating oocytes (devo), early diplotene germ cells (ed), follicular cells (fc), oocyte nuclei (nu), and ovarian unit sheath (double black arrows) are also visible. D-E) Vitellogenic oocytes (vo) occupied the terminal position in the unit. Cytoplasm is enriched in numerous small yolk spheres (stars), red stars mark the lipid accumulations, and short red arrows mark mitochondria accumulations in the cortical ooplasm. LM, epon semi-thin sections stained with methylene blue.

Fig. 10. Zone II in *Delaya* sp. GR – histology visualized by longitudinal semi-thin sections. A-D) Previtellogenic (pvo), early vitellogenic oocytes (evo), and vitellogenic oocytes (vo) are

visible. Numerous nurse cells (nc) are scattered between oocytes. Note cytophore (cy) in the form of irregular cytoplasmic strands. Double arrows mark the ovarian unit sheath, fc – follicular cells. E-F) Vitellogenic oocytes (vo) occupying the terminal positions in units and accompanying nurse cells (nc). Note nuclei of nurse cells (black arrows) within the cytophore (cy), especially nucleoli of nurse cells are well visible. fc – follicular cells; nu – oocyte nuclei. LM, epon semi-thin sections stained with methylene blue.

Fig. 11. Growing oocytes and accompanying nurse cells of *Delaya* sp. FR – ultrastructural details. A) Previtellogenic oocyte (pvo) is bigger on section than nurse cells (nc), oolemma is smooth, but no microvilli are visible. B) Early vitellogenic oocyte (evo) – microvilli started to form (arrows). C) Early vitellogenic oocyte – accumulations of mitochondria (m) close to the nucleus (nu). D-E) Early vitellogenic oocytes – accumulations of organelles and nutrients in the oocyte periphery. Inset in E – higher magnification of mitochondria. F) Fragment of lipid accumulation in the terminal (vitellogenic) oocyte. G-I) Nurse cells (nc) accompanying early vitellogenic oocytes (evo). Nurse cells are interconnected via ring canals (red arrows) to the cytophore (cy); some nurse cells have dense cytoplasm (see H and I). cy – cytophore, er – endoplasmic reticulum; evo – early vitellogenic oocyte; fc – follicular cells; G – Golgi complex; ld – lipid droplets; m – mitochondria; nc – nurse cells; nu – nucleus; ov – ovary envelope; pvo – previtellogenic oocyte; y – dense yolk granules. Arrows mark the oolemma; double arrows – oolemma with microvilli; ellipse – desmosome-like junction; red arrows – ring canals; white stars – nucleoli. TEM.

Fig. 12. Growing oocytes and accompanying nurse cells of *Delaya* sp. GR – ultrastructural details. A) Previtellogenic oocyte (pvo), no nutrients yet visible, oolemma starts to form microvilli (double arrows). B-C) Early previtellogenic oocytes. Note prominent accumulations of mitochondria and microvilli (mv). D-E) Vitelogenic oocytes accumulate organelles and nutrients. F) Nurse cells (nc) accompanying early vitellogenic oocyte (evo).

Red arrows mark the ring canal. G) A fragment of spacious cytophore (cy) containing nurse cell nuclei (nu). Such cytophores are characteristic of the distalmost part of the ovarian unit. cy – cytophore; evo – early vitellogenic oocyte; fc – follicular cells; G – Golgi complex; ld – lipid droplets; m – mitochondria; nc – nurse cells; nu – nucleus; pvo – previtellogenic oocyte; y – dense yolk granules. Double arrows mark forming microvilli; red arrows – ring canals; white stars – nucleoli. TEM.

Fig. 13. Ovisac with late vitellogenic oocytes in *Delaya* sp. GR (A-B) and *Delaya* sp. FR (C-D) – general histology visualized by LM. A) Ovisac is filled with vitellogenic (vo) and late vitellogenic oocytes (lvo). The ovisac envelope (oe) is enriched with blood vessels (bv). B) A fragment of late vitellogenic oocyte – note folds of the oocyte surface (black stars). Ooplasm is densely packed with yolk spheres (y). C) General view of late vitellogenic oocyte isolated from the ovisac. D) The same oocyte fragment showing a nucleus (nu) with a prominent nucleolus (white star). Ooplasm is densely packed with yolk spheres (y). Different staining properties of yolk spheres (light and dark blue colors) in B and C are due to the folding of the sections. Epon semi-thin sections stained with methylene blue.

Fig. 14. Ovisacs in *Delaya* sp. GR (A-C) and *Delaya* sp. FR (D-E), ultrastructural details of late oocyte organization. A) General view of the oocyte periphery. The cortical cytoplasm lacks reserve material; mitochondria (m) can be seen here. B) Higher magnification of the cortical cytoplasm. Note that among microvilli (mv), a delicate fibrous material is deposited – it is interpreted as a vitelline envelope (black stars). C) Accumulation of mitochondria (m) found in oocyte interior. D) General view of the oocyte periphery. The cortical cytoplasm is narrow, and mitochondria can be found here. Ooplasm is filled with reserve material. E) A detail of the oocyte periphery. Note a coated pit formed by oolemma. Vitelline envelope seems to be absent. gl – glycogen granules; ld – lipid droplets; m – mitochondria; mv – microvilli; y – yolk spheres. Arrow marks a coated pit; black stars – vitelline envelope. TEM.

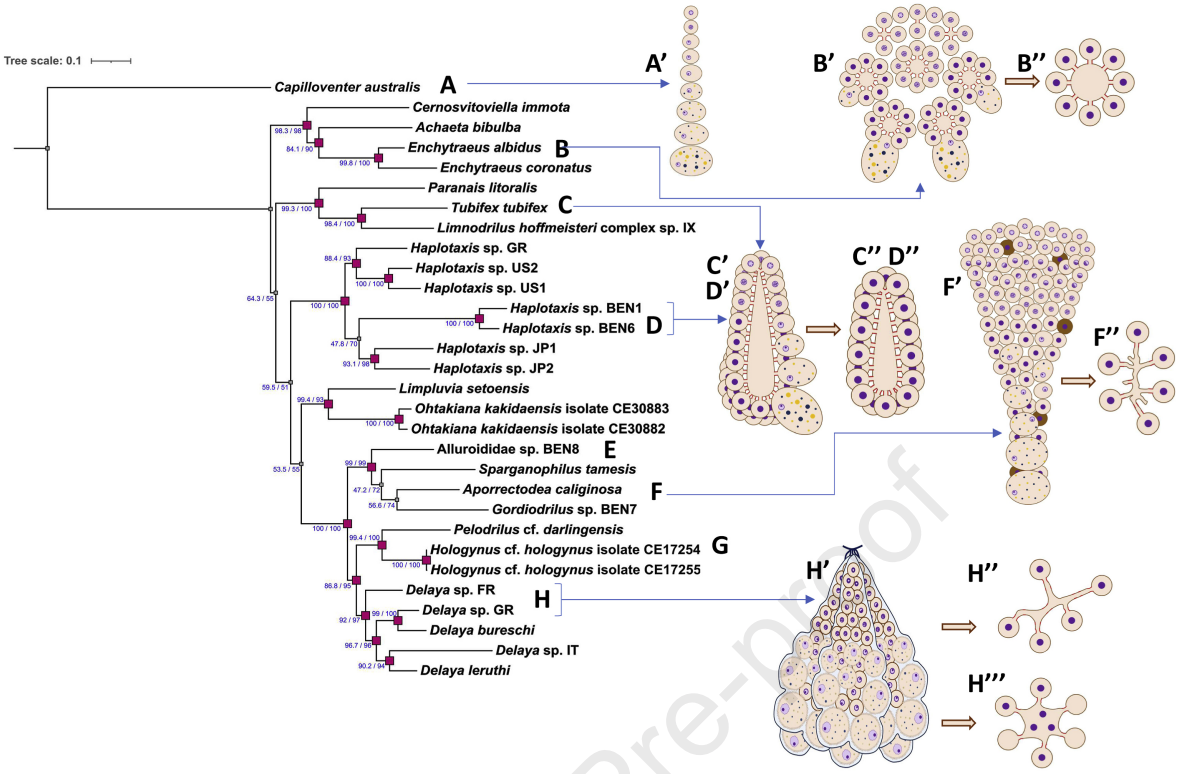
Fig. 15. A general scheme of the ovary (A), the details of the ovarian unit organization (B), and the fragment of ovisac (C) in *Delaya* sp. GR. For the details see the text; germline cysts are presented in detail in Fig. 16. Bu – cells in a bouquet stage; cy – cytophore; dgc – degenerating germ cell; ed – cells in early diplotene; evo – early vitellogenic oocytes; lvo – late vitellogenic oocyte; mi – oogonia in mitosis; nc – nurse cells; oo – oogonia; pvo – previtellogenic oocyte; vo – vitellogenic oocyte; long arrow – ovisac; short arrows – ovarian unit envelope; double short arrows – ovary attachment to the septum. Follicular cells are omitted.

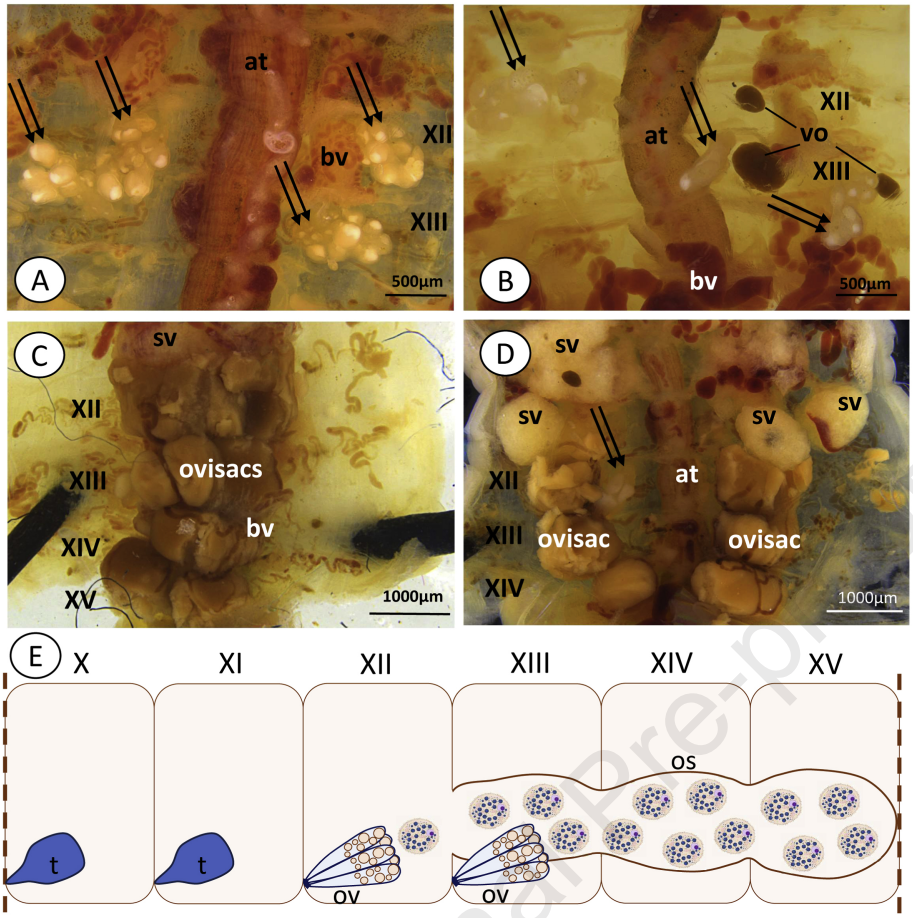
Fig. 16. Three consecutive phases of cysts functioning in *Delaya* sp. FR and *Delaya* sp GR. As a rule, each germline cell is connected to the common central cytoplasm (cytophore) via one ring canal (short arrows). Ring canals have electron-dense rims (red line); the same electron-dense material may line the cytophore membrane (marked in red in A, B, and C). A) Early cyst uniting oogonia (oo). Thin, reticular cytophore (cy) stretches between cells. Long arrow marks cytoplasmic projection. B) Cyst interconnecting early diplotene cells (ed). Cytophore (cy) is composed of long cytoplasmic strands. C) *Delaya* sp. FR cysts interconnecting nurse cells in zone II still have reticular cytophore. D) *Delaya* sp. GR cysts interconnecting nurse cells (nc) that accompany the vitellogenic oocyte (vo) in zone II. The ball-like cytophore (cy) may contain nuclei of nurse cells (dashed line arrows). For clarity, the organelles within the cytophore are omitted.

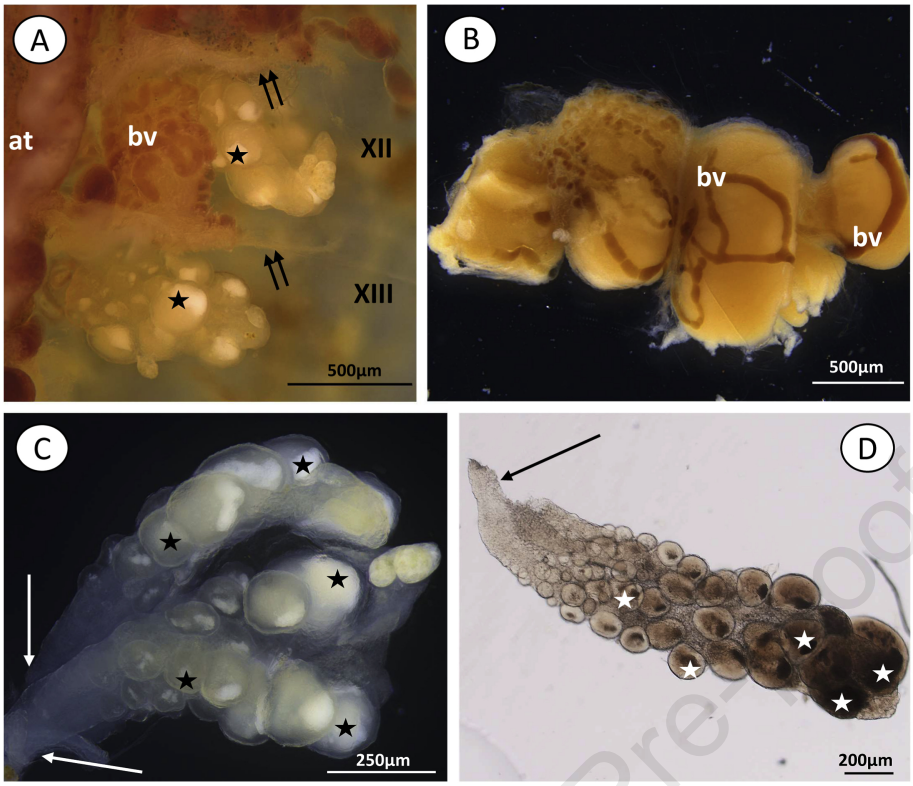
| Character | *Delaya* spFR | *Delaya* spGR | *Haplotaxis* spp[1] | microdriles[2] | Allurodidae[3] | Moniligastridae, *Drawida*[45] | Crassilcitellata[678] |
|---|---|---|---|---|---|---|---|
| Number and localization of ovaries | Two pairs in segments XII and XIII | Two pairs in segments XII and XIII | Two pairs in segments XI and XII | Usually, one pair, rarely two pairs or unpaired. Localization varies depending on the taxon, e.g., in VI, XI, XII, or XIII segment | Usually, one pair in segment XIII | Usually, one pair in segment XI | Usually, one pair in segment XIII |
| General morphology<br><br>Ovary type | Cone shaped<br><br>"Delaya" | Cone shaped<br><br>"Delaya" | Cone shaped<br><br>"Tubifex" | Different, taxon-dependent "Tubifex", "Enchytraeus", "Insodrilus", "Stylaria", "Capilloventer" | ? | Ribbon-like | Different morphology, taxon-dependent, "Dendrobaena", "Amynthas" and other |
| Functional subunits | Ovarian units | Ovarian units | Absent | In "Tubifex" and "Capilloventer" ovarian units absent. In other systems, separated cysts may function as separate units | ? | ? | Absent, egg strings may be multiplied |
| Gemline cysts with cytophore | + | + | + | + | ? | + | + |
| Number of clustering cells | ? | ? | Dozens or hundreds – multicellular cysts | Dozens or hundreds – multicellular cysts | ? | ? | Around eight, oligocellular cysts |
| Cytophore | Reticular | Reticular, then more prominent – balloon-like | Tree-like | Tree-like or balloon-like | ? | ? | Reticular |
| Nurse cells | Present | Present | Present | Present | ? | ? | Present |
| Egg type | Mesolecithic | Mesolecithic | Mesolecithic | Mesolecithic | Mesolecithic | Mesolecithic | Oligolecithic |
| Ovisacs | Well-developed with yolk-absorbing oocytes | Well-developed with yolk-absorbing oocytes | Not noticed | Present or absent, taxon dependent | Present or absent, taxon dependent | Well-developed | Usually present, poorly developed |

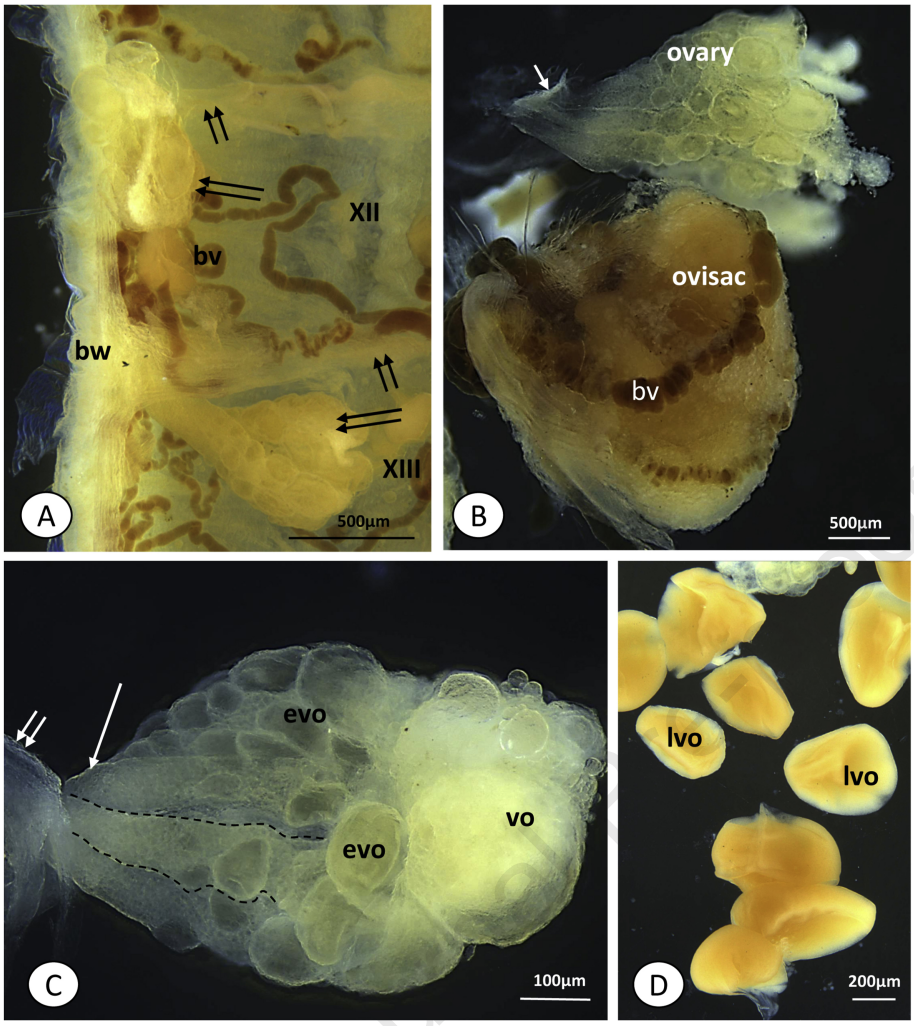
[1] Urbisz et al., 2021
[2] Timm and Martin, 2015
[3] Jamieson and Fragoso, 2024
[4] Stephenson, 1930
[5] Urbisz et al., unpublished results
[6] Stephenson, 1930
[7] Świątek et al., 2023a
[8] Raś et al., 2025

Table 1. The comparison of selected characters between the discussed taxa. For a detailed description of ovary types, see Świątek and Urbisz 2019; Raś et al. 2025. "+" – character present, "?" – no data.





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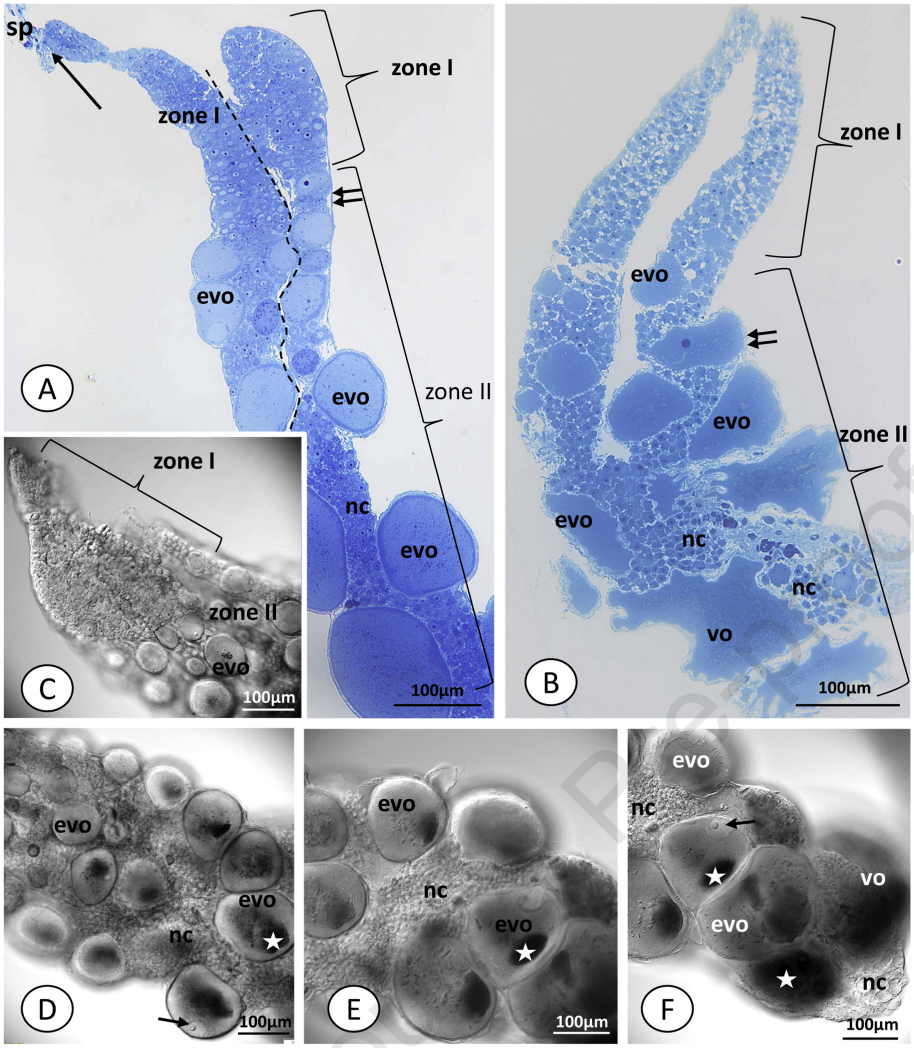

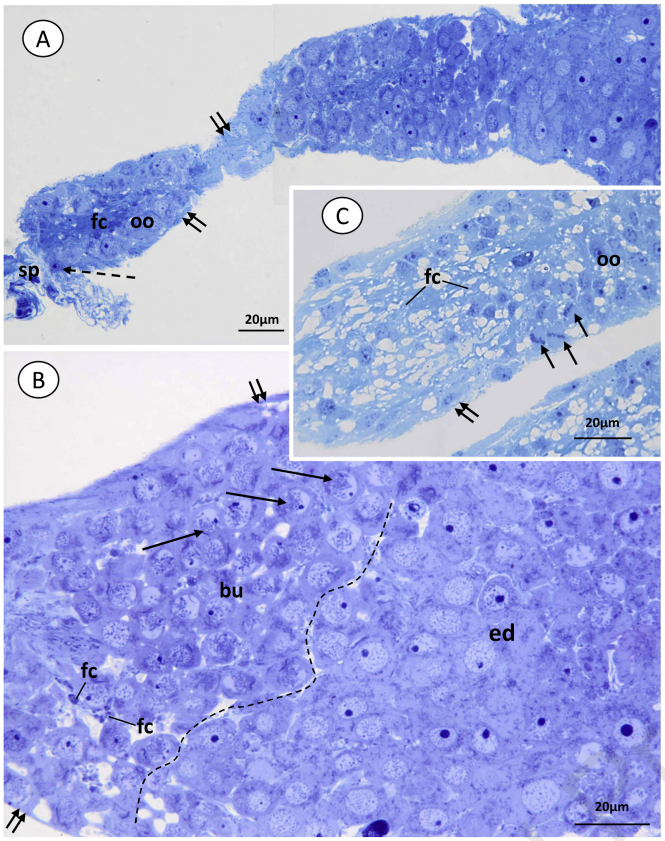

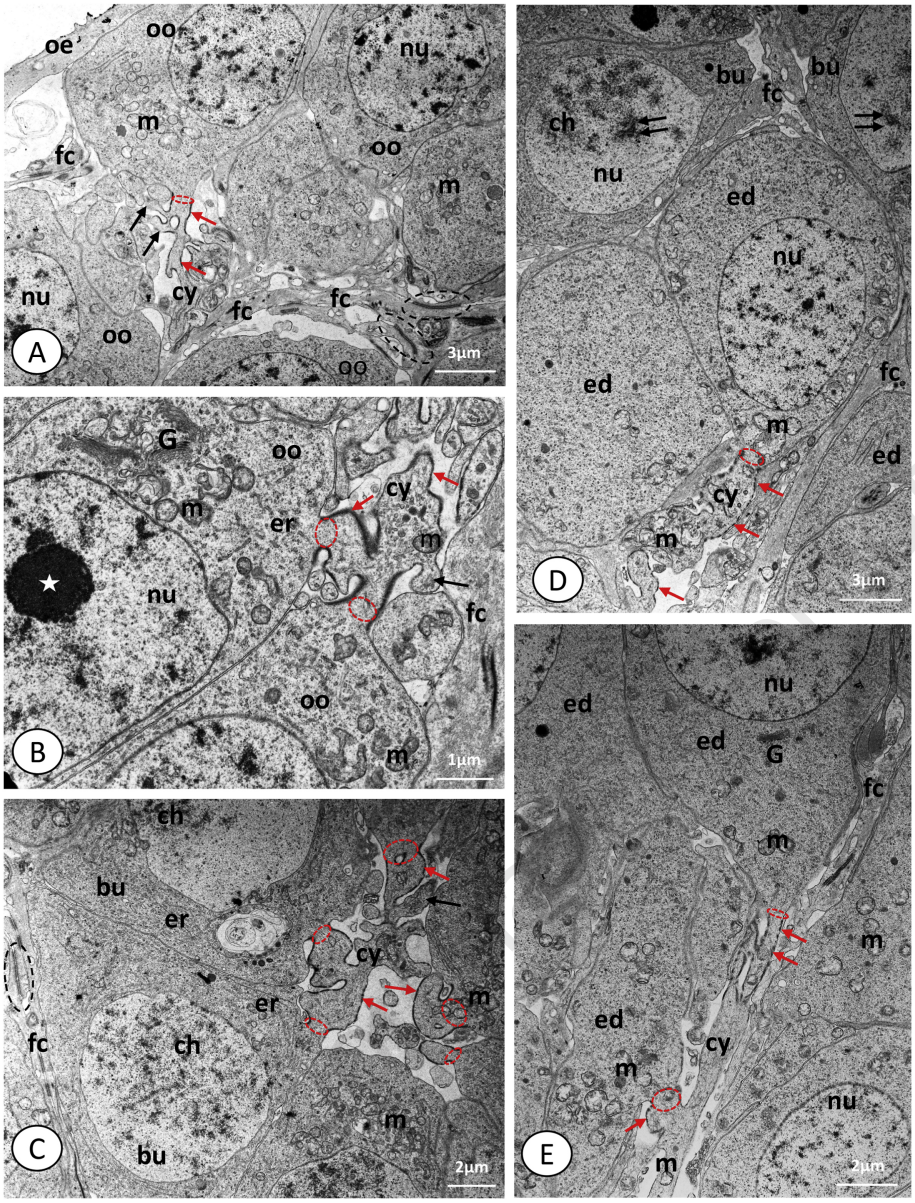
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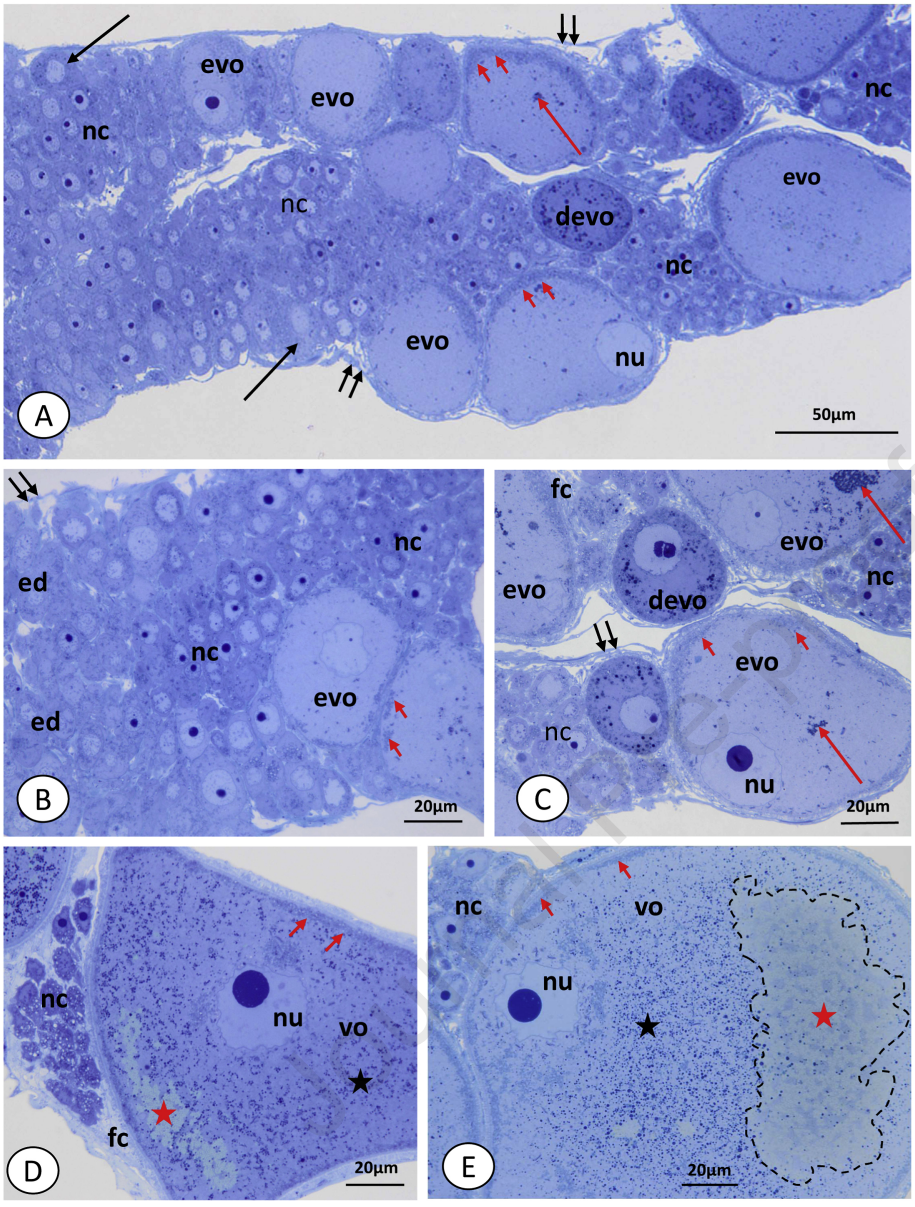
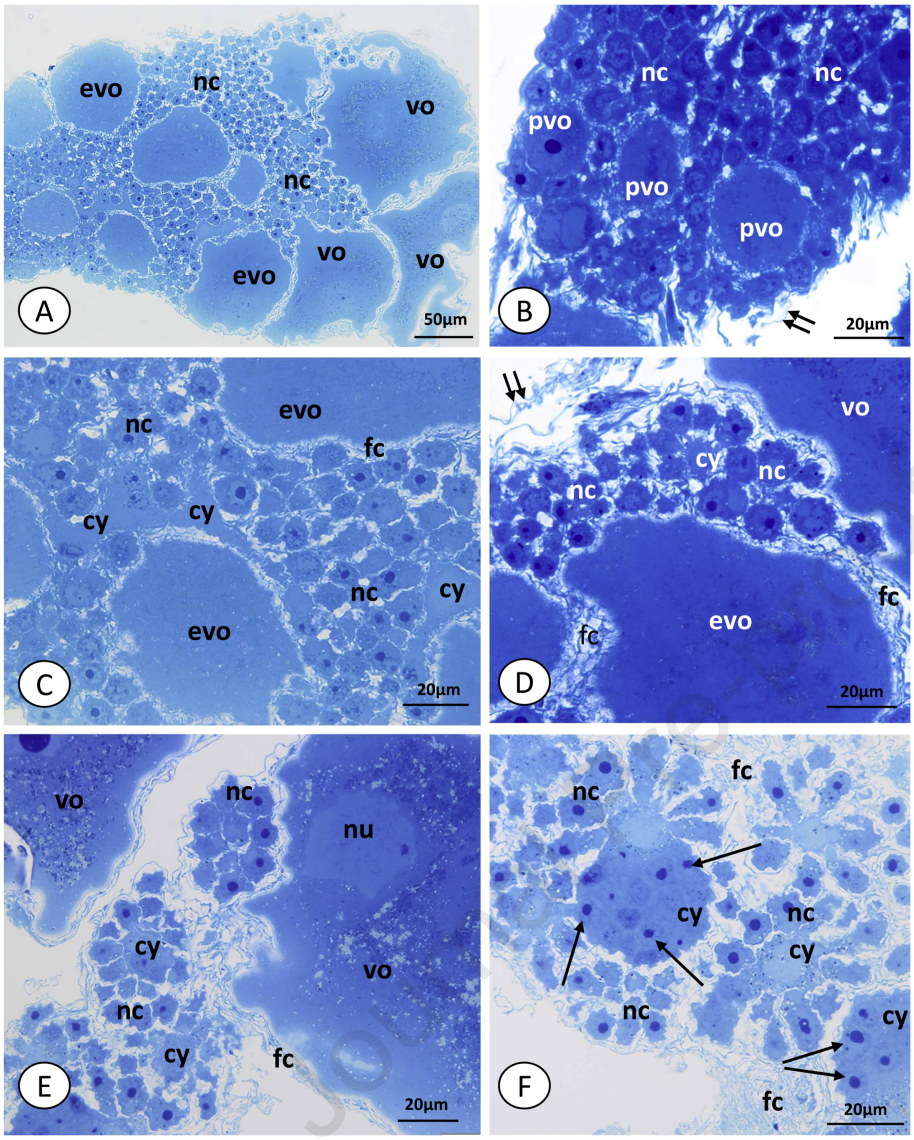
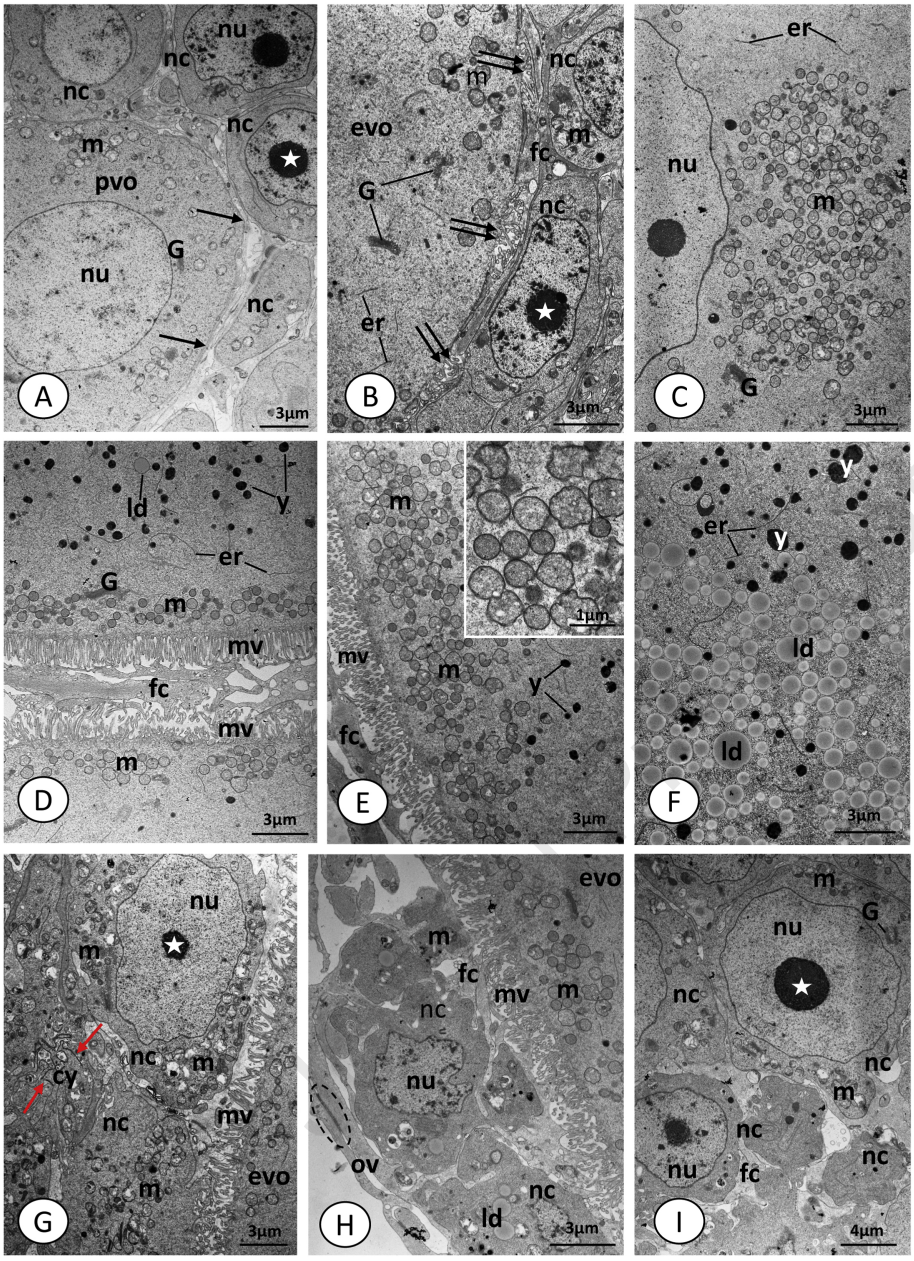
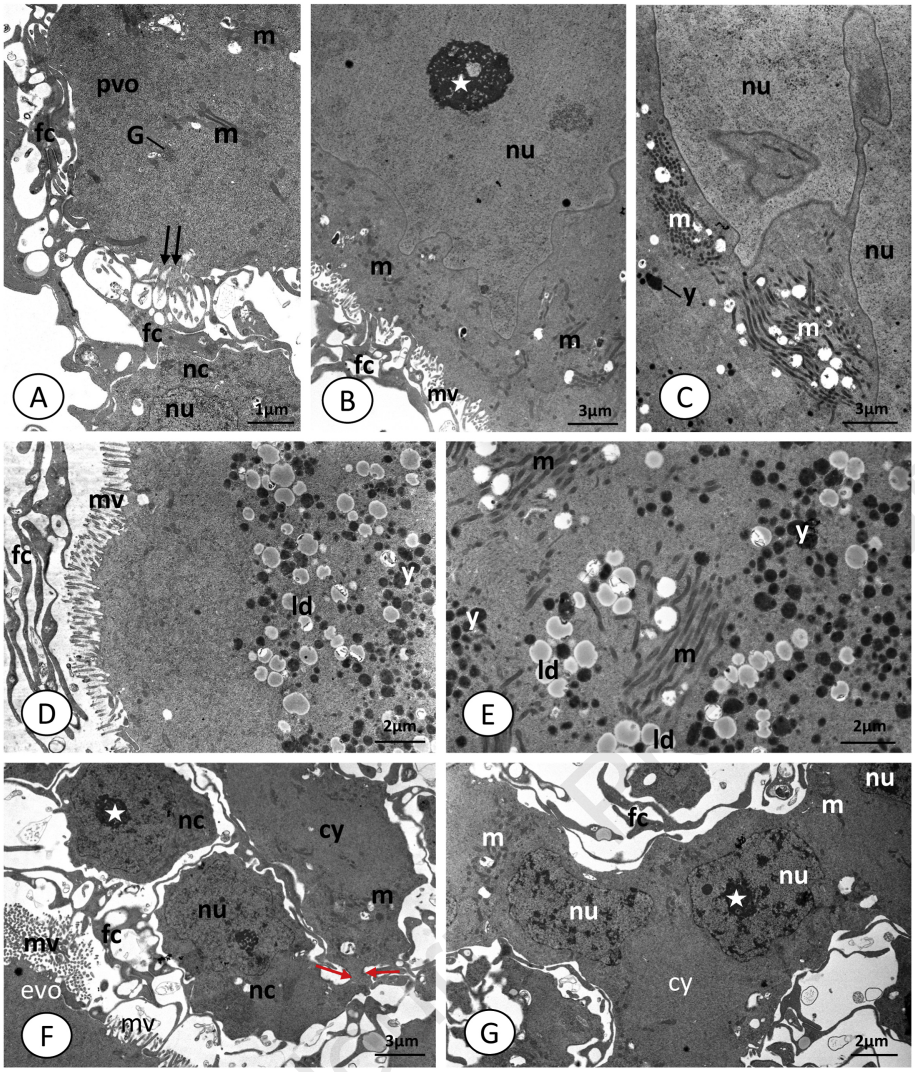
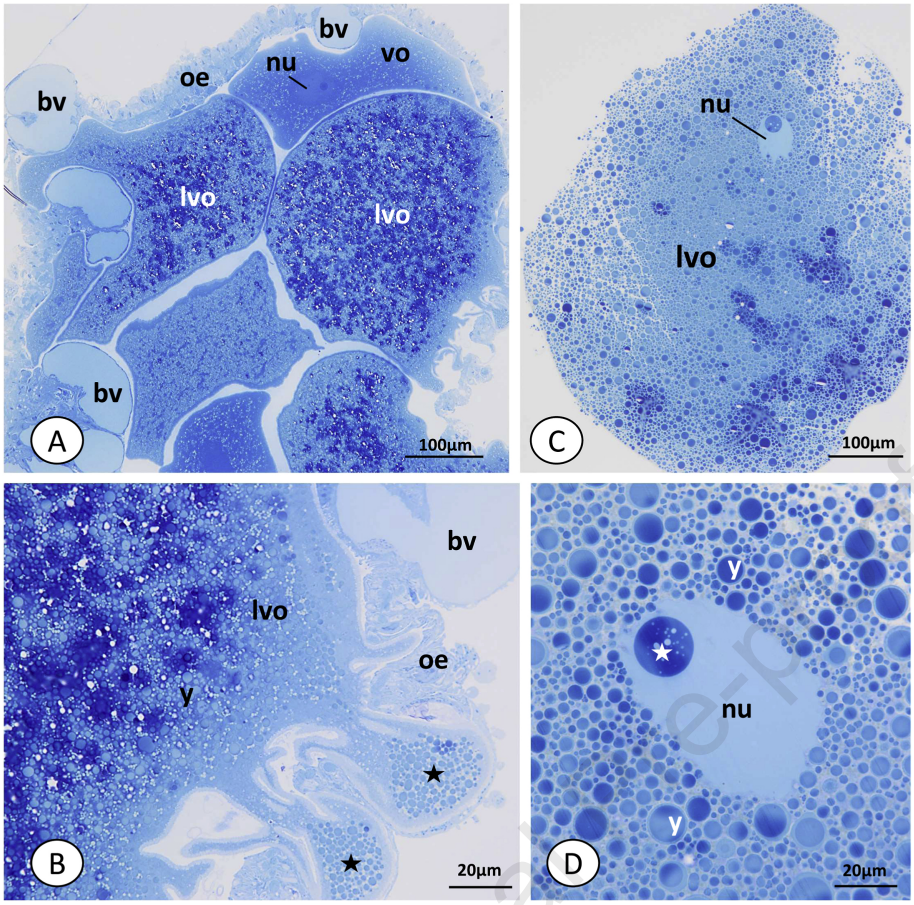
cy
m
nu
m
2µm
E

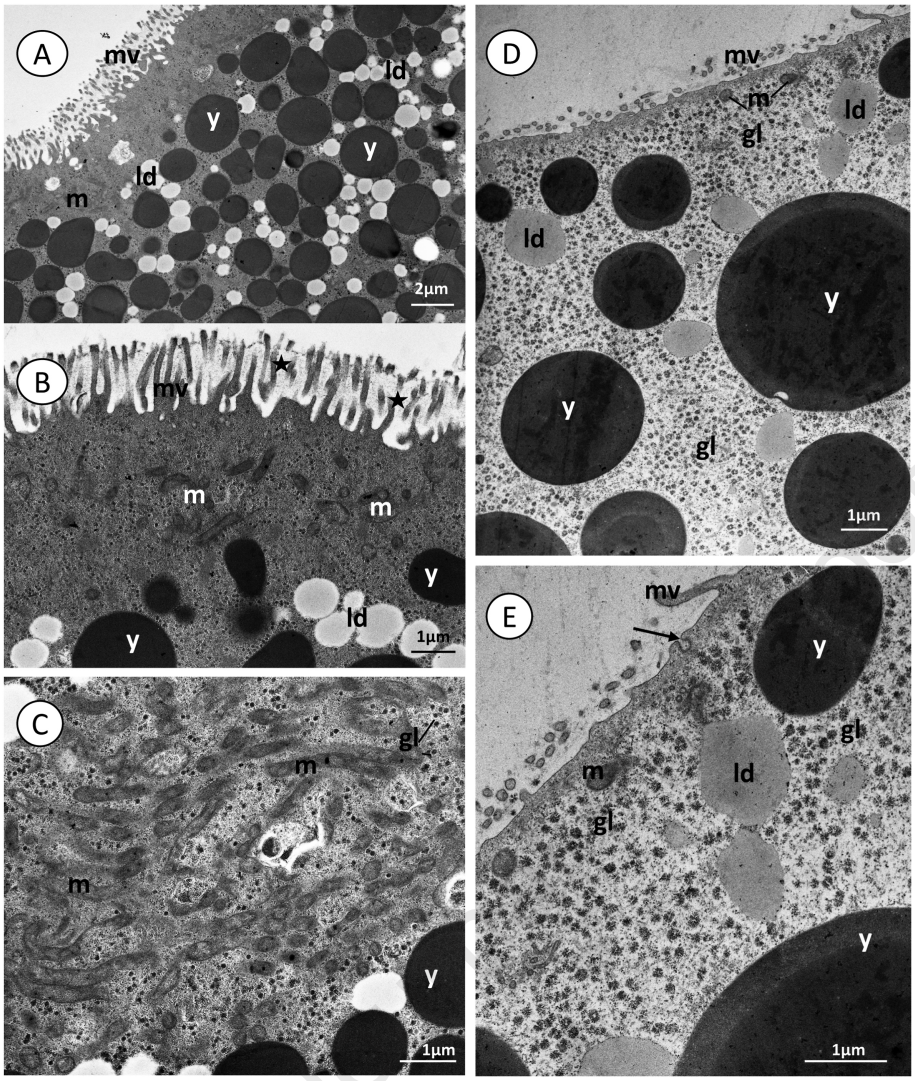

evo
evo
nc
nc
nc
evo
devo
nc
evo
nu
A
50μm
nc
ed
nc
evo
ed
B
20μm
fc
evo
evo
devo
nc
evo
nc
nu
C
20μm
nc
nu
vo
fc
D
20μm
nc
vo
nu
E
20μm


evo
nc
vo
nc
vo
vo
evo
A
50µm
nc
nc
pvo
pvo
pvo
B
20µm
nc
evo
fc
cy
cy
nc
cy
evo
C
20µm
vo
cy
nc
nc
fc
evo
fc
D
20µm
vo
nc
nu
cy
vo
nc
cy
fc
E
20µm
fc
nc
cy
nc
cy
nc
cy
fc
F
20µm


A
nu
nc
nc
nc
m
pvo
G
nu
nc
3μm
B
nc
m
evo
fc
m
G
nc
er
3μm
C
er
nu
m
G
3μm
D
ld
y
er
G
m
mv
fc
mv
m
3μm
E
m
1μm
mv
m
y
fc
3μm
F
y
er
y
ld
ld
3μm
G
nu
m
nc
m
cy
nc
mv
nc
m
evo
3μm
H
evo
m
m
fc
mv
m
nc
nu
ov
nc
ld
3μm
I
m
G
nu
nc
nc
m
nc
nc
nu
fc
nc
4μm


m
pvo
fc
G
m
fc
nc
nu
A
1µm
nu
m
m
fc
mv
B
3µm
nu
m
nu
y
m
C
3µm
mv
fc
y
ld
D
2µm
m
y
y
ld
m
ld
E
2µm
nc
cy
nu
m
mv
fc
evo
nc
mv
F
3µm
nu
m
fc
m
nu
nu
cy
G
2µm




A
mv
ld
y
y
ld
m
2µm
B
mv
m
m
y
ld
y
1µm
C
gl
m
m
y
1µm
D
mv
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gl
ld
ld
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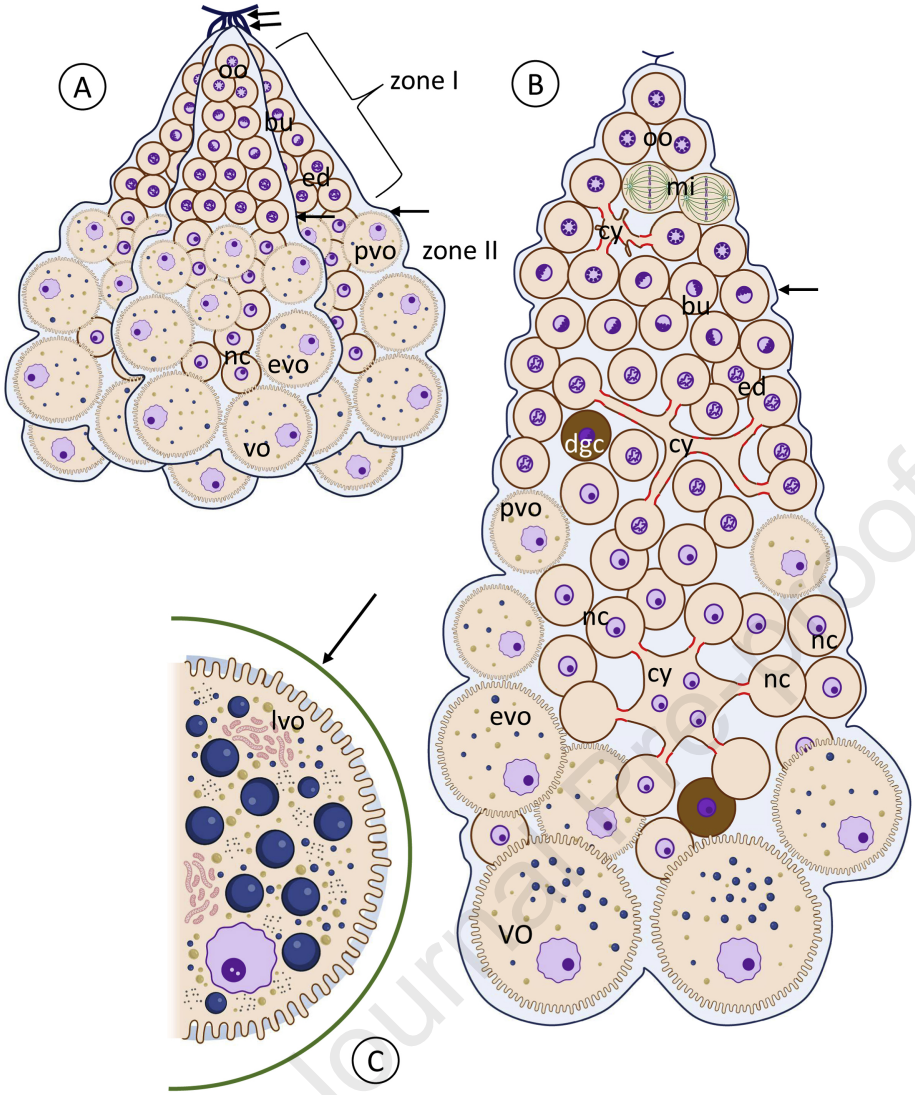
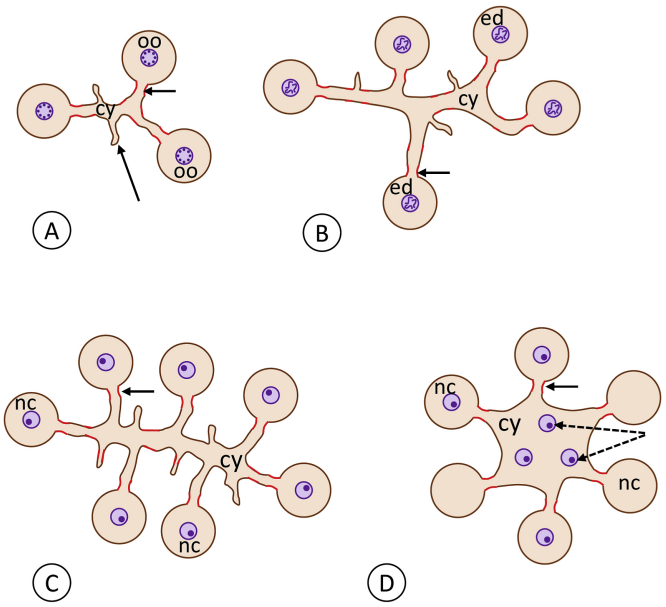
y
gl
1µm
E
mv
y
gl
m
ld
gl
y
1µm





*Delaya* sp. ovary differs structurally, leading to a new "Delaya type" classification.

Germline cyst organization in *Delaya* sp. aligns with other clitellate annelids.

Inconspicuous cytophore and the low cell number are typical of earthworms.

Ovary and oogenesis in *Delaya* sp. advanced knowledge of reproduction and phylogeny.